CONSTRUCTION OF CITRUS GENOME MAPS WITH AMPLIFIED FRAGMENT LENGTH POLYMORPHISM (AFLP) MARKERS

By

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Dedicated to the researcher and their assistants who seek the truth

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The Amplified Fragment Length Polymorphism (AFLP) marker system was used to construct a total citrus genome map based on an intergeneric backcross population of *Citrus grandis* ('Thong Dee') X USDA 17-40 (an F1 hybrid of *C. grandis* x *Poncirus trifoliata*). The map contains 337 DNA markers and a virus resistance gene (*Ctv*), and it was calculated by the JoinMap linkage analysis program. The map covers 858.6 cM of the citrus genome with 11 linkage groups, and an increased marker density of 2.5 cM/interval. A comparative analysis was conducted with the map based on AFLPs and one based on RAPD (random amplified polymorphic DNA) markers. Two maps were integrated, to generate a composite high-density citrus map with 509 markers of various types, covering a genetic distance of 1026.4 cM. The map density was compacted to 2.0 cM /interval. Map synteny analysis revealed genomic region rearrangements between major linkage groups of these two maps. Fifteen salt tolerance-related and 6 growth-related traits were selected for QTL analysis with MapQTL (JoinMap version) program. There were 36 putative QTLs found in association with growth-related traits, and 50 putative QTLs for salt tolerance-related traits. The possible genuine QTLs for those traits were analyzed further.

Marker-assisted selection was used to construct a localized genetic map for the *Ctv* gene region with AFLP markers. A subset of recombinants for *Ctv*-linked markers from a backcross population of *Citrus* and *Poncirus* were selected and subjected to CTV resistance phenotyping. Putative *Ctv*-linked AFLP markers were mapped, and the marker density in the *Ctv* region was increased. A consensus map was constructed based on three different mapping sources, which presented integrated map information of *Ctv* and a citrus nematode resistance gene region. These markers, along with those for salt tolerance, could be useful for marker-assisted selection in citrus rootstock breeding programs.

CHAPTER 1
INTRODUCTION

Citrus is the number one fruit crop grown widely throughout tropical and subtropical regions of the world. Citrus fruits have been cultivated and enjoyed for over 4000 years, and they are one of the major vitamin C sources in the diets of many people. Many chemical compounds have been identified in citrus which are proving to be beneficial to human health, in fights against birth defects, cardiovascular disease, and cancer. In the U.S., significant citrus industries exist in Florida, California, Texas and Arizona, and the Florida citrus industry is very important to the state economy, with an estimated value of over $ 6 billion.

Since the late 19th century, citrus breeding and cultivar development have become very important to the citrus industries of the world. Traditional genetic study and cultivar development of citrus are very difficult because of several breeding impediments, including self-incompatibility, nucellar embryony (Soost and Cameron, 1975), long generation time and juvenility related character expression, large tree size, and the lack of rapid and effective hybrid seedling screening procedures (Gmitter et al., 1992). A citrus genome map, consisting of easily scored molecular markers closely linked to economically significant loci, would be a powerful tool for citrus cultivar improvement, by allowing early screening of progeny for key traits and manipulation of quantitative traits based on marker assisted selection, and further manipulation of economically important traits for genetic advance by gene cloning and transformation.

Generation of genetic linkage maps for plants, traditionally based on morphological, biochemical and behavioral markers, has been accelerated by the use of protein and DNA markers. Isozyme and restriction fragment length polymorphism (RFLP) markers have been used to generate linkage maps in number of species (Torres et al., 1985; Tanksley, 1983; Helentjaris et al., 1986; Jarrell et al., 1992; Durham et al., 1992), including plants where little information had been known previously, such as citrus (Jarrell et al., 1992; Durham et al., 1992). Random amplified polymorphic DNAs (RAPDs), another type of DNA marker, based on random amplification of genomic DNA with single primers of arbitrary nucleotide sequence, have been found useful for generating or expanding linkage maps in plant species (William et al. 1990; Klein-Lankhorst et al. 1991; Michelmore et al. 1991; Reiter et al. 1992; Tulsieram et al. 1992; Torres et al. 1993; Warburton et al., 1996). In *Citrus*, a total genomic map consisting of 109 RAPD markers along with 51 previously mapped RFLP and isozyme markers (Cai et al. 1994), and localized linkage maps for the CTV resistance gene (Gmitter et al. 1996) and for the citrus nematode resistance gene region (Ling et al. in press), were generated. With the extensible utilization of RAPDs, other types of PCR-based DNA markers with specific advantages were derived and used in citrus genetic studies such as SCARs (Deng et al. 1997), RGCs (Deng et al. 1999), and ISSRs (Fang et al. 1997). Like many other fruit crops, citrus has encountered many different diseases, pests, and various environmental stresses throughout their growing regions in the world. The existing gene pool of citrus harbors gene(s) for resistance to different diseases and pest, and QTLs that are responsible for tolerance to different physiological stress such as cold and salinity. A high-density genetic map of citrus will serve as a fundamental tool for locating these beneficial genes in the citrus genome relative to their linked molecular markers. Although citrus genome maps have been developed previously, there still are many regions remain to be filled with additional molecular markers.

Amplified fragment length polymorphism (AFLP) is a relatively new DNA fingerprinting technique (Zabeau and Vos 1993; Vos et al., 1995). This DNA marker system is based on PCR amplification of selected restriction fragments of digested total genomic DNA, and separation of labeled amplified products by denaturing polyacrylamide gel electrophoresis. In many aspects, it is a combination of RFLP and RAPD techniques. A great advantage of the AFLP technique is that it allows simultaneous identification of a large number of amplification products. AFLPs have been used to develop genome maps in many plant species (Wang et al., 1997; Qi and Lindhout et al., 1997; Lu et al., 1998; Mackill et al., 1996; Becker et al., 1995). AFLP possibly is the most efficient marker system that can be used to generate genome maps in a relatively short time, or to identify markers that are tightly linked to disease resistance genes in certain genomic regions of plants (Ballvora et al., 1995; Thomas et al., 1995; Simons et al., 1997; Lu et al., 1998). The technical details of the AFLP development are described in the following chapter.

The first objective of this study was to develop a high-density citrus genome map based on AFLP markers, combined with a few SCARs and RGCs, and integrated with previously mapped RAPDs, RFLPs, and isozymes. The intergeneric backcross population of 'Thong Dee' [*Citrus grandis* (L.) Osb.] X USDA 17-40 [*C. grandis* x *Poncirus trifoliata* (L.) Raf.], previously used to construct RFLP and RAPD-based citrus genome maps (Durham et al., 1992; Cai et al., 1994), was used to construct the AFLP-based citrus genome map. A second objective was to enrich the citrus tristeza virus resistance gene (*Ctv*) region and citrus nematode resistance gene (*Tyr1*) region with AFLP markers, along with previously determined *Ctv*- and *Tyr1*-linked SCAR, RAPD and RGC markers. To serve this purpose, a selected group containing 101 individuals recombinant for *Ctv*-linked markers, from a intergeneric backcross population of DPI 4-5 [*Citrus grandis* (L.) Osb.] X USDA 17-47 [*C. grandis* x *Poncirus trifoliata* (L.) Raf.], was used to construct a high-resoultion consensus map of the *Ctv* region. The individuals of this selected recombinant group have been subjected to CTV resistance phenotype evaluation, and a portion available (50%) of the CTV resistance phenotype data was used to position *Ctv* relative to closely linked markers.

The following chapter is a review of the literature, including information on the use of molecular markers in plant genetic studies. Other topics reviewed are the general utilization of AFLP markers in genetic mapping, tagging disease resistance genes in plants, and available mapping software programs. The three chapters following the literature review focus on the construction of the total citrus genome based on AFLP markers, the construction of an integrated high-density citrus genome map to identify QTLs for growth- and salt tolerance- related traits, and the construction of a consensus map for *Ctv* and *Tyr1* regions. Important points and results will be discussed in each chapter. The final chapter provides a summary of the research and some conclusions relevant to the use of the AFLP markers and future research prospectives.

CHAPTER 2
REVIEW OF THE LITERATURE

Introduction

The major objective of this research was to construct a high-density total genome map for citrus based on a recently developed molecular marker system, amplified fragment length polymorphism (AFLP), along with other preexisting marker types and QTL regions. A second objective of this research was to use AFLP markers to saturate the region containing the citrus tristeza resistance gene (*Ctv*), and to construct a new localized map for *Ctv* region.

For the citrus genetic mapping study, two intergeneric populations derived from *Citrus* x *Poncirus* backcrosses were used. *Poncirus trifoliata* is in the genus *Poncirus*, closely related to *Citrus*. *P. trifoliata* is one of the most important gene resources for citrus disease resistance breeding programs. It confers resistance to several important pathogens and pests of citrus, such as citrus tristeza virus (CTV), citrus nematode, *Phytophthora* root rots, and tolerance to cold. Flying Dragon, one strain of *P. trifoliata*, trasmits a gene for tree size control in citrus when used as rootstock (Roose, 1990; Wheaton et al., 1991). These important traits can be transmitted to *Citrus* by sexual hybridization. Because many undesirable fruit characters of *P. trifoliata* also are transmitted by sexual hybridization, the hybrids of the intergeneric cross between these two genera are mainly used in rootstock development. Three traits that have economic significance in citrus improvement, and may be inherited as a single genes, are CTV resistance (Yoshida, 1985; Garnsey et al., 1987, Gmitter et al., 1996), citrus nematode resistance (Hutchison, 1985; Ling et al., 1996) and nucellar embryony (Cameron and Soost, 1979).

In this chapter, a review of citrus genetics and its relevance to citrus breeding is presented. Then, general information is presented on the various types of molecular markers including isozymes, restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), simple sequence repeats or microsatellites (SSR), sequence characterized amplified region (SCAR), resistance gene candidate (RGC), and amplified fragment length polymorphism (AFLP). The application of these molecular markers in genetic mapping of plants will be discussed. Finally, information on computer software packages, used to analyze marker segregation data and for map construction, will be considered.

Citriculture and Citrus Breeding

Citrus Genetics and Breeding

Citrus is grown throughout tropical and subtropical region of the world where there is sufficient water for growth and winter temperatures are warm enough for tree survival. Citrus is the number one fruit crop in the world in terms of annual production (Citrus Summary 1997-1998, Florida Agricultural Statistics Service, Department of Citrus). Since late 19th century, breeding and cultivar improvement have been active in many regions of the world including Florida. Despite vigorous efforts, the majority of citrus scion and rootstock cultivars have not originated from planned breeding schemes, but rather originated from chance seedlings or bud mutations (Hodgson, 1967). The gene pool of *Citrus* is substantial, because most of the citrus species are interfertile. However, the gene pools for the most important economically species are rather narrow, especially for *Citrus sinensis* [L.] Osb. (sweet orange) and *C. paradisi* Macf. (grapefruit)(Gmitter et al., 1992). Improvement of citrus cultivars has included many aspects, and the demands are gradually increasing as more and more of the health benefit components are discovered. Horticultural characters including fruit characters (color, flavor, size, shape, season of maturity); disease and pest resistance; and tolerance to physical stress (cold, salt, drought etc.) are primarily considered by plant breeders for cultivar improvements. Several closely related genera are sexually compatible with citrus, and some intergeneric hybrids have been fertile (Swingle and Reece, 1967). Diverse genetic resources exist that could be utilized for cultivar improvement, or for the expansion of existing citrus gene pools. Sexual hybridization and selection in some way are capable of integrating valuable alleles from diverse intrageneric germplasm or some closely related genera, to creating desirable phenotypes in citrus.

However, many aspects of citrus biology have been strong impediments to genetic advances. Most of the citrus species have long periods of juvenility which can last five years or more (Soost and Cameron, 1975). Additionally, many tree and fruit characteristics are atypical in young seedling and flowering stages, compared to maturity. Because of the long juvenility, much time is required for growing seedlings to their fruiting stage, before any evaluation and meaningful selection can take place. The numbers of the hybrid progeny which can be subject to evaluation and selection is limited by the large tree size at maturity, because the land space and tree care expenses for growing the plants is always a limitation. It would be very advantageous if marker-assisted selection could take place at the seedling stage, once molecular markers for important traits are available. Like many other fruit tree crops, most of the traits in citrus are quantitatively inherited. The progeny segregate for such traits in a continuous fashion, so understanding their mode of inheritance is much more difficult by traditional genetic study approaches without using DNA markers to investigate at the genome level. It is almost impossible to locate the genomic regions responsible for these traits by traditional genetic studies.

Adventive embryony has significantly prevented true hybrid production from cross-hybridization of citrus clones. The nucellar embryos derived from adventive embryony compete with the zygotic embryo for space and nutrients during the seed development in polyembryonic clones, resulting in loss of the zygotic embryo (Gmitter et al., 1992). Thus, hybrid populations of sufficient size are difficult to obtain. This problem has ruled out many citrus clones as breeding parents, although they carry desirable traits. Furthermore, gametophytic self- and cross-incompatibility exist in some of the mandarins (*C. reticulata* Blanco) and their hybrids, and among the pummelos (*C. grandis* [L.] Osb) (Soost and Cameron, 1975). Because of the self- and cross-incompatibility system in the genus, some cultivars and selections that could be useful breeding parents are excluded. Because of these factors, citrus genetic studies also have been hindered, resulting in a lack of genetic information on many important traits. Additionally, timely and cost efficient screening methods that can be used in the young seedling stages are lacking.

Molecular Markers in Linkage Maps

In the early twentieth century, genetic mapping studies were limited to morphological markers that are under single gene control (Landry et al., 1987; Tanksley et al., 1989). Morphological markers usually can be easily scored for little cost, but their expression can be influenced by environmental factors or epistatic interactions. A detailed linkage map requires many detectable mutants, but this condition is limited to very few species of annual crops. Moreover, the populations used in mapping usually are F_2 or backcross populations; therefore, mapping for morphological markers is limited to those species with a large number of visible mutants and available testing lines (Lander et al., 1987).

Alternatives to morphological marker-based linkage maps are provided by several different types of biochemical markers that have been applied to citrus and other crops. These alternatives have covered a broad range of techniques, for example, a crude assay for polyphenol oxidase-catalyzed browning of young shoot extracts (Esen and Scora, 1975). Other more useful markers that have been used with citrus and other plants include leaf isozymes that can be visualized on starch gels (Torres, 1983; Torres et al., 1985; Jarrell et al., 1992; Durham et al., 1992), DNA sequence polymorphisms detected by probing restricted genomic DNA with labeled DNA sequence clones (RFLPs, or restriction fragment length polymorphism), and polymerase chain reaction generated fragments (RAPDs, or random amplified DNA sequences), and by the latest DNA finger-printing techniques derived from combination of the latter two marker systems, generating restricted amplified DNA fragments (AFLPs, amplified fragment length polymorphism).

Protein markers, generally isozymes representing different molecular forms of the proteins that exhibit the same enzymatic specificity, can be studied by gel electrophoresis (Goodenough, 1984). Most of the isozyme alleles are codominant in nature without epistatic interaction so that genotypes can be determined directly. Many isozymes are not tissue or developmental stage specific. Only a small amount of tissue is needed, and such experiments are relatively inexpensive and not time consuming. Genetic studies based on isozyme markers have been reported in citrus (Torres et al., 1978; 1983; Moore and Castle, 1988; Durham et al., 1992; Roose et al., 1989), and many other crops. A high rate of polymorphism has been reported for various enzymes in apple when they were used for the identification of scion varieties (Weeden and Lamb 1985; Mendendez et al., 1986; Korban and Bournival 1987), and rootstock varieties (Vinterhalter and James 1986; Samimy and Cummins 1992). A few close linkages have been established between isozymes and single genes coding agronomic traits, such as the self-incompatibility gene *S* in apple (Battle et al., 1995), the apple scab resistance gene V_f (Manganaris et al., 1994), and mildew resistance gene Pl_w (Battle and Alston 1996). Isozyme markers were used to detect alleles for the nematode resistance gene *Mi* in tomato (Rick and Forbes, 1974) and single disease resistance genes in lettuce (Landry et al., 1987). Isozyme markers were also used to detect linkage between markers and quantitative trait loci in common bean (Zhang et al., 1992). Isozyme markers have greatly enhanced genetic studies for many organisms that lack morphological markers (Tanksley, 1983). Isozyme markers also have been valuable as anchor markers to establish a reliable correspondence between genetic maps built from different progenies (Chevreau et al., 1999). However, the numbers of isozyme are limited, because they represent only small part of the total genome.

The technology of restriction fragment length polymorphism (RFLP) is one of the milestones in the development of genetics in this century, which has led to a huge wave of progress in genetics (Zhang and Wing, 1997). RFLPs denote the differences in molecular weight of homologous DNA fragments created by treating DNA with restriction enzyme (Bostein et al., 1980; Hudson, 1982). RFLPs arise from variation present in DNA sequences, either by base pair changes, rearrangements, or insertions/deletions (Reiter et al., 1992). The fragments produced by digesting genomic DNA with restriction enzymes can number in the millions in a continuous range in sizes. Through hybridization, labeled DNA probes hybridize to blotted DNA restriction fragments, and the fragment homologous to the probe can be detected as polymorphic fragments (Southern, 1975). Because cloned DNA is required to construct probes, most of the probe sequences should be single copy or lower copy number sequences. This characteristic has made RFLP markers a stable and reliable molecular marker system for genetic studies. RFLP markers are useful because they are codominant and inherited in Mendelian fashion, they are phenotypically neutral and rarely exhibit epistasis or pleiotropy, and they are detectable in both adult and juvenile plants (Tanksley, 1983;

Tanksley et al., 1989). The number of RFLPs that can be identified is virtually unlimited. Therefore, RFLPs are one of the most powerful molecular marker systems for genetic studies. It has been used to develop detailed linkage maps with one or a few segregating populations (Helentjaris et al., 1986; Landry et al., 1987; Tanksley et al., 1989; Durham et al., 1992; Jarrell et al., 1992; Xu et al., 1994; Boivin et al., 1999). RFLPs have been efficiently used to identify markers linked to disease resistance genes and quantitative trait loci of many annual crops such as rice, tomato, potato, lettuce, beans and wheat (Young et al., 1988; Paterson et al., 1988; Messeguer et al., 1991; Ronald et al., 1992; Nodari et al., 1993; Martain et al., 1993; Eastwood et al., 1991; Kreike et al., 1996; Redoña and Mackill 1996; Rouppe van der voort et al., 1998). In fruit crops, RFLPs were used to identify the scab resistance gene *Vf* in apple (King et al., 1998), and mapping of cold tolerance in citrus (Cai et al., 1994). RFLPs were also useful in germplasm analysis of soybean (Powell et al., 1996) and cultivated potato (Milbourne et al., 1997).

Because RFLPs have higher reliability, locus specificity, and often represent single or lower copy of the genome sequences, they are often chosen as anchor markers for the integration of different linkage maps with other type of markers from different populations, and to allow synteny studies (Becker et al., 1995; Lin et al., 1995; Dufour et al., 1996; Dufour et al., 1997). For example, the use of RFLP clones as map anchors has revealed that chromosome segments of rice and maize (Ahn and Tanksley, 1993), and rice and wheat (Kurata et al., 1994) contain DNA markers in a very similar order. RFLP marker maps were populated with RAPDs and AFLPs to construct high-density genetic maps for soybean (Keim et al., 1997), and sorghum (Boivin et al., 1999). RFLP markers are also the most suitable markers used in the construction of a physical map of rice using BACs (bacterial artificial chromosomes) (Zhang and Wing 1997). Evenly distributed RFLP markers can give greater advantages over other types of markers when used as anchor markers for physical and genetic integration, because single copied probe sequences can be used directly to hybridize with various large insert clones for correct contig overlapping analysis.

Technology of polymerase chain reaction (PCR) (Mullis et al., 1986; Saiki et al., 1988; Williams et al., 1990) is another milestone technology development for molecular genetic study. A random region of DNA can be amplified through the use of the short, arbitrarily chosen oligonucleotide primers and thermostable DNA polymerase (Williams et al., 1990). The polymorphic DNA fragments detected, based on PCR amplification using random primers, were termed RAPD (random amplified polymorphic DNA) markers. RAPD analysis has been used as alternative method in targeting DNA sequences for genetic characterization and mapping. The polymorphism detected by RAPDs can reflect genomic DNA sequence base variation at the primer binding site, differences in genomic DNA structure in the region of amplification, or both (Reiter et al., 1992).

There are several advantages associated with RAPDs over other type of markers. RAPD markers can be produced from any species without any prior DNA sequence information (Williams et al., 1990). Only a small amount of the genomic DNA is required (10-100ng) for PCR amplification. In many cases, the DNA purity requirement is also less restrictive than other marker systems. The RAPD analysis does not require

Southern hybridization, nor the use of radio-labeled probes, and the techniques used are relatively simple and rapid. Detection of RAPD markers is not limited in the genomic region with low copy number, so DNA polymorphism can be detected in regions containing highly repetitive sequences. A disadvantage of RAPD markers is that they usually segregate in dominant, rather than codominant fashion; polymorphisms detected by RAPDs usually appear as presence or absence of the bands, thus heterozygotes cannot be distinguished from the homozygous dominant class. Despite this major disadvantage, the RAPD technique has became the most widely used molecular marker system for genetic analysis determination of many species.

RAPD markers have been used for cultivar identification in wheat (Devos et al., 1992), barley (Shin et al., 1990), rice (Williams et al., 1991), rose (Torres et al., 1993), and numerous other crops. More important and valuable applications of RAPD markers are their use in construction of genome maps, because of their simplicity and low development costs. RAPD marker-based genetic linkage maps have been developed for lettuce (Michelmore et al., 1991), tomato (Martin et al., 1991; Tanksley et al., 1992), *Arabidopsis* (Klein-Lankhorst et al., 1991; Paran et al., 1993; Reiter et al., 1992), apple (Hemmat et al., 1994), citrus (Cai et al., 1994), peach (Chaparro et al., 1994; Rajapakse et al., 1995) and many other crops. By applying bulked segregant analysis (BSA) (Michelmore et al., 1991), RAPD markers were developed to target genes for resistance to *Pseudomonas syringae* in tomato (Martin et al., 1991), downy mildew resistance gene in lettuce (Paran et al., 1991), bacterial blight resistance gene in rice (Ronald et al., 1992), and stem rust resistance gene in oats (Penner et al., 1993). RAPD markers also have been used in fruit tree crops to identify genes of economic interest. In peach, RAPD markers were mapped relative to genes controlling flesh color, flesh adhesion to the seed, and flesh texture (Warburton et al., 1996). In citrus, RAPD markers were used to target the citrus tristeza virus resistance gene (Gmitter et al., 1996; Fang et al., 1998), citrus nematode resistance genes (Ling et al., inpress), drawfing gene (Cheng and Roose 1995), and gene regions for cold and salt tolerance (Cai et al., 1994; Tozlu, 1997)

Other PCR-based markers systems such as SCARs (sequence characterized amplified regions) (Paran and Michelmore, 1993) and SSR (simple sequences repeats, microsatellites) (Tautz, 1989; Weber and May 1989) have been used in plant genetic studies. SCAR markers are developed by cloning and sequencing the RAPD products, designing longer specific primers based on that selected RAPD fragment sequence, and amplifying DNA under stringent conditions (Paran and Michelmore, 1993). SCARs have been reported in several plants, including lettuce (Paran and Michelmore 1993; Witsenboer et al., 1995), common bean (Adam-Blondon et al., 1994), grape (Resich et al., 1996), raspberry (Parent and Page, 1995), and citrus (Deng et al., 1997). The conversion of RAPDs into SCAR markers has significantly increased specificity, reliability, and reproducibility of the PCR assays, and it also allows development of PCR based codominant markers (Deng et al., 1997). Thus it is often used in targeting specific gene regions.

Microsatellites, or SSR markers, were used initially in mammalian genetic studies, because of high levels of polymorphism associated with the SSR loci in mammalian genomes (Caetano-Anolles et al., 1991; Cornall et al., 1991; Dietrich et al., 1992; Serikawa et al., 1992). Recently, microsatellite markers have been developed for plant species, especially where RFLP polymorphism is low (Akkaya et al., 1992). Microsatellite markers have been developed in crop plants including soybean (Akkaya et al., 1992; Morgante and Olivieri, 1993; Morgante et al., 1994; Rongwen et al., 1995), rice (Wu and Tanksley, 1993; Yang et al., 1994), barley (Saghai Maroof et al., 1994), maize (Seniro and Heun, 1993), *Arabidopsis* (Bell and Ecker 1994), grape (Thomas and Scott, 1993; Thomas et al., 1994) and citrus (Kijas et al., 1997). Studies in plant species have indicated that microsatellite markers are highly polymorphic and can generate a larger number of markers in a single gel analysis. Therefore, it is one of the marker systems suitable for genetic mapping in most plant species. However, a significant disadvantage of microsatellite markers is that identifying and mapping these loci is expensive and time-consuming. Nonetheless, the overall high informativity of the SSR marker system has made them increasingly popular in plant genetic studies.

Considerable progress has been made recently in cloning disease resistance genes from several plant species (Bent 1996; Jones and Jones 1997) by means of transposon tagging and/or map-based cloning after many years of effort. Many of these genes have similar sequence motifs, even though they determine resistance to very different pathogens, including viruses, fungi, and bacteria. Some of those isolated genes including *RPS2*, *RPM1*, *I2*, *N*, *RPP5* and *L6* share the sequences characteristic of the ATP/GTP binding sites or nucleotide binding site (NBS) of the P-loop proteins (Hammond-Kosack and Jones 1997). The presence of conserved domains among the cloned resistance genes presents the opportunity to clone numerous additional resistance genes from diverse species by PCR with degenerate oligonucleotide primers corresponding to the conserved motifs, based on known nucleotide or amino acid sequences of the specific proteins (Shen et al., 1998). Based on PCR techniques, resistance gene candidate (RGC) markers are developed from known genes of interest, expressed cDNA, transposable elements, and expressed sequence tags (EST). Such markers can be used to identify new resistance genes across species. RGC markers provide a higher possibility of finding genes or gene-like sequences within the resistance gene cluster region, and of isolating the novel sequences representing functional genes. The development of RGC markers provides a direct way to screen, identify, and isolate genes from genomic DNA or cDNA libraries, and from BAC or YAC clones. Degenerate oligonucleotide primers have been used to isolate NBS-containing sequences from soybean (Kanazin et al., 1996; Yu et al., 1996), potato (Leister et al., 1996), rice (Leister et al., 1998), lettuce (Shen 1998) and *Arabidopsis thaliana* (Aarts et al., 1998). In citrus, four DNA fragments derived from NBS motifs, associated with the citrus tristeza virus resistance gene (*Ctv*) and a major citrus nematode resistance gene (*Tyr1*), were identified, mapped and cloned. The translated amino acid identity of these markers ranged from 20% to 46.7% when compared with known resistance gene sequence in other species including *Arabidopsis thaliana* and tobacco (Deng et al., in press). Also of significance, the RGC markers have provided new opportunities to investigate gene-for-gene interactions between plants and pathogens, and to classify various resistance genes. Further, RGCs may help to understand the path of resistance gene evolution in different plant species over time. As more resistance genes are cloned and sequenced, more pathways and end products are being determined and isolated, and sequence information becomes increasingly available in the gene data base. The further utilization of RGC markers should be much more frequent in the time to come.

AFLP DNA Fingerprinting Technology for Genetic Mapping

Recently, a DNA fingerprinting technique called amplified fragment length polymorphism (AFLP) has been developed (Zabeau and Vos 1993; Vos et al., 1995) for genetic studies. The technology is based on the amplification of selected restriction digest fragments of total genomic DNA by PCR, and separation of labeled amplified products by denaturing polyacrylamide gel electrophoresis. This technique has combined both RFLP and PCR-based marker systems together to achieve maximum revelation of DNA polymorphisms.

The principles of AFLP

AFLP technology is a DNA fingerprinting technique that combines strategies of classical hybridization-based fingerprinting with PCR-based fingerprinting. It is based on the selective amplification of subsets of genomic restriction fragments using PCR. DNA is digested with restriction endonucleases, and double-stranded DNA adapters are ligated to the ends of the DNA fragments to generate template DNA for amplification. In this way, the sequence of the adapters and the adjacent restriction sites serve as primer binding sites for subsequent amplification of the restriction fragment by PCR. Selective nucleotides extending into the restriction fragments are added to the 3' ends of the PCR primers such that only the subset of the restriction fragment are recognized. Only the restriction fragments in which the nucleotides flanking the restriction site match the selective primer nucleotides will be amplified. The subset of amplified fragments is analyzed by denaturing polyacrylamide gel electrophoresis to generate the DNA fingerprints. When the DNA fingerprints of related samples are compared, common bands as well as different bands are observed. The differences, or DNA polymorphisms, can be scored. Polymorphism obtained by restriction cleavage can result from alterations in the DNA sequences including mutations abolishing or creating a restriction site, and insertions, deletions, or inversions between restriction sites. The AFLP technique involves three major steps: (1) Restriction endonucleases digestion of DNA and ligation of adapters; (2) Amplification of the restriction fragments; (3) Gel analysis of amplified fragments.

Restriction endonucleases digestion

To prepare an AFLP template, high quality genomic DNA must be isolated (Dellaporta et al., 1983) and digested with a combination of two restriction endonucleases simultaneously. This step generates the required subset of fragments for adapter sequence ligation and subsequent amplifications. The two restriction endonucleases used normally are a "rare cutter" which has a six base recognition site and another "frequent cutter" with a four base recognition site. When six base and four base cutters are used together in a suitable genome size range, these enzymes generate small DNA fragments that will amplify well and are in the optimal size range (<1Kb) for separation on denaturing polyacrylamide gels. For instance, "rare cutter" *Eco*RI and "frequent cutter" *Mse*I are often used to carry out the digestion. Due to primer design and amplification strategy, these *Eco*RI/*Mse*I fragments are preferentially amplified rather than *Eco*RI/*Eco*RI or *Mse*I/*Mse*I fragments (Vos et al., 1995). The success of the AFLP technique is dependent upon the complete restriction digestion. Therefore, high quality genomic DNA which is intact and without contamination of nucleases or inhibitors is required.

Ligation of the adapters

Following the heat inactivation of the restriction enzymes after complete genomic DNA digestion, genomic DNA fragments are ligated to *Eco*RI and *Mse*I adapter sequences to generate template DNA for amplification. Each of these double strand adapter sequences is ligated to their own enzyme cutting site and the flanking variable genomic DNA sequences, and each adapter sequence serves as the primer binding site for the restriction fragment. By using such strategy, many DNA fragments can be generated and amplified without having prior sequence knowledge.

Amplification reactions

PCR amplifications are performed in two consecutive reactions. The first reaction is the preamplification. Genomic DNA is initially amplified with a pair of primers, one primer with sequences matching the *Mse*I adapter and cutting site sequences plus one extended selective nucleotide (N+1); and the other primer with sequences matching the *Eco*RI adapter and cutting site sequences with no extended selective nucleotide (N+0).

The PCR products of preamplification reaction are diluted and subsequently used as templates for selective amplifications with two AFLP primers: the *Mse*I selective primer contains three selective nucleotides (N+3) and the *Eco*RI selective primer contains two selective nucleotides (N+2). Usually the *Eco*RI selective primer is labeled with ^{33}P or ^{32}P before amplification. The pair up ratio of *Mse*I selective primer and *Eco*RI selective primer is normally maintained as 9:1 in the amplification reaction. This two step amplification results in consistently cleaner and more reproducible DNA fingerprintings.

The number of selective nucleotides is the most important factor in determining the number of restriction fragments amplified. A second factor that influences the number of amplified fragments is the C and G composition of the selective nucleotides. In general, increasing the C/G number of selective nucleotides in the selective amplification primers, decreases the number of DNA fragments amplified. The complexity of the genome also influence the number of restriction fragments amplified (Vos et al., 1995).

Analysis of amplified fragments on denaturing polyacrylamide gels

Products from the selective amplification are separated on a 6-8% denaturing polyacrylamide gel. The resulting bands can be observed from autoradiography, and the polymorphism can be analyzed either manually or using analytical software. When scored visually, AFLPs behave as dominant markers, similar to RAPDs, although some of them can be scored as codominant markers with appropriate computer software. Overall, the procedure of AFLP technique is more time-consuming and expensive than RAPDs, but the total number of loci that can be scored in a single gel is much greater.

Modifications on the AFLP system

There are two technical modifications in the AFLP system that can provide optimum detection of polymorphisms. The first modification is the application of suitable restriction enzyme combinations to carry out the genomic DNA digestion. The AFLP polymorphism segregation patterns are influenced by the distribution of the restriction enzyme recognition sites, because the recognition site of a particular restriction enzyme in the genome of a given species segregates as other genetic elements in the population. Thus by manipulating different restriction enzyme combinations to carry out the digestion of the genomic DNA, different numbers and distributions of the polymorphism for map construction result. The second modification is the alteration of adapter sequences ligated to the restriction enzyme digested fragments, and therefore the restriction enzyme primers will also need to be modified. By applying different adapter sequences and their correspondent primer pairs in the PCR amplification, the selective amplification can aim to a much more defined genomic region according to the adapters and initial primer pair design. For instance, by designing adapters and primer pair sequences according to a gene sequence, RGC class sequences, or expressed sequences tags (ESTs), one can amplify digested fragments in a more determined fashion, and achieve the higher possibility of identifying fragments belonging to certain gene families or gene clusters in a genomic region.

Genome Mapping and Targeting Disease Resistance Genes with AFLP Markers

A great advantage of the AFLP technique is that it allows simultaneous identification of a large number of amplification products. It is an efficient technique to generate large numbers of markers for genetic mapping, compared with other DNA marker system (van Eck et al., 1995; Mackill et al., 1996; Keim et al., 1997). AFLP markers have been developed very rapidly for the construction of genetic maps in many crops including barley (Qi and Lindhout 1997; Powell et al., 1996), melon (Wang et al., 1997), rice (Mackill et al., 1996; Nandi et al., 1997; Subudhi et al., 1998), sorghum (Boivin et al., 1999), lentil (Eujayl et al., 1998), potato (Ballvora et al., 1995), peach (Lu et al., 1998), and citrus (Ling et al., in preparation). Because it is possible to generate a large number of markers for a given population, AFLPs have been used to construct large scale high-density genetic maps for the integration with physical genome maps in some plant species including sorghum (Klein 1999), *Arabidopsis thaliana* (Zhang 1999) and rice (Lightfoot 1999). In addition to construction of genetic maps, the AFLP technique has been efficiently used to identify specific genes. AFLPs were used to identify markers that cosegregated with tomato *Cf-9* gene for resistance to *Cladosporium fulvum*, and the genomic clones encompassing the *Cf-9* gene were identified further by *Cf-9* cosegregating AFLP markers (Thomas et al., 1995). In *Populus*, AFLPs were used to identify markers tightly linked to the *Mer* locus that conferred resistance to leaf rust, *Melampsora larici-populus* (Cervera et al., 1996). The AFLP technique was employed to identify markers tightly linked to the *Mlo* locus that conferred resistance to powdery mildew fungus, *Erysiphe graminis f.*sp. *hordei*, and to produce a localized high-resolution genetic map in barley (Simons et al., 1997). AFLP-based RNA fingerprinting was used to identify genes that are differentially expressed (Bachem et al., 1996; Money et al., 1996). AFLP markers were used to enrich the potato *Gro1* gene regions on the chromosome VII (which confers resistance to root cyst nematode, *Globodera rostochiensis*) (Ballvora et al., 1995), and to enrich the potato *R1* allele region on chromosome V (which confers resistance to *Phytophthora infestans*) (Meksem et al., 1995). In *Arabidopsis*, AFLP markers were used to build a high-resolution map within 3cM in the *TRN1* gene region (Cnops et al., 1996).

The AFLP technique is able to provide greater genome coverage by employing the combination of two restriction enzymes. Therefore it is advantageous to use AFLP markers to construct high-density genome maps for analyzing quantitative traits loci (QTL) in many crops. The advantage becomes even greater when AFLP markers are integrated with other types of markers in the genome, because this allows comparative mapping studies to be conducted. In barley, AFLP markers were combined with RAPDs and RFLPs, and 12 QTLs were located in the genome (Powell et al., 1997). By the same methodology, a major QTL responsible for submergence tolerance in rice was localized in chromosome 9, and other QTLs associated with submergence tolerance were detected on chromosomes 6, 7, 11, and 12 (Nandi et al., 1997).

AFLP technology has also been widely used to study the genetic diversity of plants (Maughan et al., 1996; Travis et al., 1996; Zhu et al., 1998), to differentiate bacterial strains (Janssen et al., 1996; Lin et al., 1996), and to detect genetic variation in fungi (Majer et al., 1996). In citrus, AFLP markers have been used to identify the somatic hybrids when other marker systems have failed to detect polymorphism between parents and fusion products (Grosser, personal communication).

Citrus genome mapping

With the advance of molecular marker techniques, genetic mapping has become important in basic genetic studies of plants, as well as for cultivar improvement. The generation of linkage maps of plant genomes, traditionally based on morphological and biochemical markers, has been accelerated by the use of DNA markers. A well defined linkage map, consisting of genetically characterized traits of importance and easily scored polymorphic marker loci spaced throughout the genome, can give valuable assistance to plant breeders because it provides a timely and relatively inexpensive way to identify individuals possessing desirable combinations of beneficial alleles without direct screening or progeny testing. In a saturated linkage map, markers that are tightly linked to useful genes may be discovered and serve as indicators for the presence or absence of the desirable alleles among segregating hybrid progeny.

Citrus is one of the most attractive fruit crops for conducting linkage analysis. Citrus is a diploid with a low haploid chromosome number, ($n = 9$) and a relatively small genome (with estimated 3.89×10^9 base pairs, 1C=0.62 pg (Guerra 1984)). The genus *Citrus* is highly polymorphic. Interspecific hybrids can be produced, as well as some intergeneric hybrids. Restriction fragment length polymorphism (RFLP) markers have been used to generated linkage maps in many plant species, including citrus (Durham et al. 1992; Jarrell et al. 1992) where few prior linkage relationships had been determined previously. An extended core map of citrus containing 109 RAPDs and 51 previously mapped RFLP and isozymes was developed with total length of 1192 cM, and it was estimated to cover 70-80% of the citrus genome (Cai et al., 1994). In another citrus genome mapping scheme, trinucleotide microsatellites were integrated into a citrus RFLP and isozyme map, demonstrating the utility of this marker type for genetic analysis within an intergeneric cross (Kijas et al., 1997). Besides the total citrus genome map construction, there are several localized citrus maps produced for disease resistance genes using various types of molecular markers (Gmitter et al., 1996; Deng et al., 1997; Mestre et al., 1997; Fang et al., 1998; Ling et al., in press), and for other genes controlling important horticultural traits (Cheng and Roose 1995). However, there are still many horticulturally important genes and quantitative trait loci that remain to be identified and located onto the citrus genome. With progress in map-based cloning and physical mapping development in various crops, advanced manipulation of genes of agronomic importance is becoming a reality. The high-density genetic map with important genes and QTLs positioned relative to their genetically linked molecular markers will provide access for positional cloning and advanced manipulation of those genes.

It would be beneficial to produce a higher-density citrus genome map as a framework for more useful genes and QTLs to be located more precisely in the future. To achieve the major objective of this research, we have adapted the new marker system, AFLP, to construct a new high-density citrus genome map primarily based on AFLP markers. Bulked segregant analysis strategy was used in another intergeneric backcross population to identify *Ctv*-linked AFLP markers and to construct a new saturated consensus map for the *Ctv* gene region with other types of markers as well.

Computer Programs for Linkage Analysis and Map Construction

Several computer programs are available for examining linkage relationships and producing maps. These computer programs including LINKAGE (Lathrop and Lalouel, 1984), LINKAGE-1 (Suiter et al., 1983), MAPMAKER (Lander et al., 1987), JOINMAP 1.3 version (Stam, 1993) and JOINMAP Version 2.0 (Stam and Van Ooijen, 1995). These programs are all based on the maximum likelihood method. LINKAGE was the first program capable of doing multipoint analysis and of analyzing quantitative genetic data. It is suitable for a wide range of applications in linkage studies. LINKAGE-1 was designed for linkage analysis using a variety of segregating progeny, including F_2 and backcross populations (Suiter et al., 1983). The program can analyze dominant (3:1 and 1:1 expected ratios) and codominant (1:1, 1:2:1 and 1:1:1:1 expected ratios) genes as segregating loci. Goodness-of-fit is determined by chi-square analysis. Contingency chi-square test was employed to analyze independent assortment between all pair of loci. The recombination frequency and its standard error (SE) are calculated using maximum likelihood formulae.

MAPMAKER was specifically designed for more accurate genetic mapping, and it is probably the most widely used program in plant genetic mapping studies. Linkage relationships can be determined by multipoint linkage analysis for two and three generations of natural populations, backcross populations, and open-crossed populations

(Lander and Bostein, 1989). The likelihood values are calculated by using the Lander-Green algorithm, which make it feasible to study a large number of loci simultaneously. MAPMAKER divides a set of markers into linkage groups using two-point analysis and computes the possible genetic order of each group, which will be compatible with three-point data. Subsequently, markers are examined by multipoint analysis to resolve any loci order differences (Lander et al., 1987). For each locus and each interval between loci, MAPMAKER displays the "relative lod-likelihood" value to support the placement of the locus in that position, and the best order of all known loci can be deduced for each linkage group. Finally, MAPMAKER displays the genetic distance in centimorgan (cM) and recombination frequencies for each interval. The recombination frequencies in the linkage map can be converted to centimorgan distance by either Kosambi (Kosambi, 1944) or Haldane (Haldane, 1919) functions. The Haldane's map function assumes absence of interference between crossover in meiosis, whereas the Kosambi's map function assumes a certain degree of interference. For most of the higher organisms, Kosambi function results in a better overall fit of recombination data.

JOINMAP was developed for construction of integrated genetic maps using raw data from F_2, backcrosses, or recombinant inbred lines (RILs). The linkage data collected from different experiments can be combined and analyzed by this program. JOINMAP has been designed for non-interaction use, which means the position of markers needs no interaction from the user, but to some extent user's interactions are taken into consideration. For example, the user may supply information in a separate file, the so called "fixed sequences" of the markers. In this case, JOINMAP will try to produce a map does not contradict any of these “fixed sequences”. The method of “calculating” a map (evaluating the order of the markers) in JOINMAP is slightly different from other linkage analysis programs. For a given order of markers, an adapted version of Jensen & Jorgensen’s method of least squares is used (Jensen & Jorgensen, 1975, rather than the chi-square method. A likelihood ratio is calculated to indicate the goodness-of-fit. The best fitting order is established by trial-and-error searching of the parameter space. When a new marker is added to the map, that map region containing this newly added marker along with previously mapped markers is rearranged in a moving interval until no better solution can be found. Estimates of recombination frequencies from raw data are obtained by using the EM algorithm, to obtain maximum likelihood estimates. JOINMAP produces the best fitting map distances between markers, as well as the cumulative distance, with the chosen map functions (Haldane’s or Kosambi’s map functions). JOINMAP Version 1.3 can only deal with a maximum of 150 markers in a data set. This appears to be significant limitation of the program when encountered with a large marker data set, such as is possible now with AFLPs.

The currently used of JOINMAP Version 2.0 (Stam and Van Ooijen, 1995) has extended the ability of the program to meet growing challenges of larger mapping data sets. The range of population types that can be processed is greatly extended, and up to five hundred markers can be analyzed in a single linkage group. Overall, the mapping processes have been significantly accelerated. In JOINMAP Version 2.0, the various tasks in a mapping project are performed by separate programs called “modules”. There are four core modules in JOINMAP 2.0: JOINMAP linkage group assignment module-

JMGRP; JOINMAP data file splitting module-JMSPL; JOINMAP recombination estimation module-JMREC; and JOINMAP map construction module-JMMAP.

JOINMAP can only construct a map for a set of markers one linkage group at a time. Running the JMGRP module is the first phase of conducting JOINMAP analyses, which assigns markers to linkage groups. The JMGRP module assigns markers to linkage groups based on LOD scores and recombination estimates for pairs of markers. Once a particular linkage group has been defined, the original data file will be split into separate files corresponding to defined linkage groups by running JMSPL module. The second phase of the JOINMAP is to calculate pairwise recombination frequencies and the corresponding LOD scores by running JMREC module for each of the linkage groups. The results of the computation is written to a "pairwise data files" for each linkage group. Finally, these "pairwise data files" are used as input for the map construction module, JMMAP, the core of the JOINMAP package. The recombination frequency and modified LOD scores are taken from "pairwise data file" to produce a linkage map. The map construction involves two classes of computational tasks including calculating the "cost function", and finding the best fitting order of markers. The cost function is the weighted least square of differences between "direct" map distance and "calculated" map distances (Lander and Botstein, 1989). The direct map distances correspond to the recombination frequency values in the "pairwise data file"; calculated map distances result from minimizing the "cost function". The goodness-of-fit is calculated and expressed as the chi-square value. The map is assembled sequentially for each newly added marker. Chi-square was calculated for every newly assembled marker set to determine the best fitting.

A number of positions in the map region where the marker is likely to fit are examined. The best fitting position then is chosen from several candidate positions. The criterion for deciding which marker is to be placed next onto the map is its summed LOD score with markers assembled in the map previously. The JMMAP module determines the first pair of markers to be mapped by the tight linkage (LOD score) of these two markers, and the significant linkage of this marker pairs to at least two other markers, in such a way that four markers are taken into consideration for one pair of marker's linkage determination. In addition to the core modules introduced above, JOINMAP 2.0 also has a number of utility modules that can be used for data inspection, data checking, and data manipulation.

There are a number of methods developed to map quantitative trait loci (QTL), controlling quantitatively measured phenotypes segregating in experimental populations. In general, the techniques detect QTL by finding correlation between the inheritance of particular genetic markers and variation in the phenotype for each individual in the population. The extensive linkage analysis program MAPMAKER/QTL is one frequently used QTL analysis program. MAPMAKER/QTL shares the same data file with MAPMAKER, and it is designed to map genes controlling quantitative phenotypes. MAPMAKER/QTL is able to provide support for "interval mapping", allowing full exploitation with genetic linkage information. MAPMAKER/QTL can determine the putative QTLs based on calculated LOD scores, and provide a measure of support for particular hypothesis. The algorithms used in MAPMAKER/QTL are based on the assumption that phenotype values are normally distributed, so data are often transformed to fit this assumption more closely. The order of genetic markers and the distance between those markers in LINKAGE-1 and MAPMAKER can be calculated and displayed compatibly with MAPMAKER/QTL. The program examines the entire linkage group to search for putative QTLs, normally at 2 cM intervals. In the result, the shape and the height of the "log-likelihood surface" is displayed, the highest log-likelihood peak indicating the most probable position of the putative QTLs.

Another QTL analysis program which is compatible JOINMAP 2.0 is MAPQTL (tm) Version 3.0 (Van Ooijen and Maliepaard, 1996). MAPQTL (tm) Version 3.0 is able to detect QTLs in an integrated mapping situation, and is able to handle a wide range of mapping population types, including backcross, F_2, RILs, doubled haploid and full-sib populations of a cross-pollinated species. This program can apply interval mapping method (Lander and Botstein, 1989; Jansen, 1993), nonparametric mapping method (Van Ooijen et al., 1993) and the mapping method based on multiple-QTL models (MQM) (Jansen, 1993; 1994). The MAPQTL (tm) Version 3.0 is able to calculate the likelihood of a segregating QTL with segregation information of all linked markers, each of which may follow a different segregation type with two and up to four alleles (Maliepaard and Van Ooijen, 1994). With MQM mapping, markers can be used as cofactors to absorb the effects of nearby QTLs, thereby increasing the power for mapping other segregating QTLs (Jansen, 1993; Van Ooijen 1994). In nonparametric mapping, there are no assumptions being made for the probability distributions according to how the quantitative trait should behave. The nonparametric mapping method uses Kruskal-Wallis rank sum test (Lehmann, 1975) to carry out one-way analysis of variance. The Kruskal-

Wallis rank sum test examines and ranks all the individuals according to the quantitative trait, the ranks are based on statistic test using classification by the genotypes. The loci with the tightest linkages to the QTL were presented by a gradient of the test statistic in the linkage group harboring the segregating QTLs. The power of the test depends on the degrees of freedom; as a consequence, when codominant markers are combined with dominant markers, the dominant loci may show a lower significance level even they are more closely linked to the QTL.

Overall, MAPQTL (tm) Version 3.0 has overcome the incompatibility between JOINMAP and the previously existing QTL mapping program. It is able to locate QTLs in the linkage map in a joined manner, thus QTL mapping can be conducted under the situation in which large numbers of markers are generated, and maps are integrated from different experiments. This program is easy to use, extremely fast, and produces clearly arranged output of the mapping results.

Summary

Molecular genetic technology has provided circumventions to overcome some of the technical difficulties of genetic studies and cultivar improvement of citrus. Genetically mapped DNA markers can be useful in studying the inheritance of traits, including QTLs, and variation among populations. Genome maps may provide tools for plant breeding programs by indirect marker-based progeny screening (Durham et al., 1992; Jarrell et al., 1992). Citrus is one of the important fruit crops on which genetic studies have been conducted, including the inheritance of the disease resistance and physical stress tolerance, and identification of horticulturally important QTLs. Citrus cultivar improvement holds great economic potential if more beneficial genes can be introduced to current or newly developed cultivars.

Citrus genetic studies have been conducted more intensively since the molecular markers have become available. The first linkage maps of citrus were produced with isozyme markers and RFLP markers (Torres et al., 1985; Durham et al., 1992; Jarrel et al., 1992). Later with availability of RAPD markers and other PCR-based markers, citrus genetic mapping has been greatly enhanced, as genes responsible for diseases resistance, environmental hardiness, and horticultural characters were identified and mapped (Cai et al., 1994; Gmitter et al., 1996; Cheng and Roose, 1996; Ling et al., in press). Citrus maps are largely extended with RAPD markers. However, the genetic study of many traits still faces the limitation of long juvenility; therefore, many traits including those controlled by QTLs remain to be identified and mapped onto the citrus genome. A high-density genome map constructed with molecular markers can provide the structure for locating newly-identified genes and QTLs when later their phenotypes are available. Moreover, a high-density citrus map can also provide easy access to integrate the relative gene positions into a physical map of the citrus genome, and thereby lead to advanced gene manipulations.

Using AFLP technology combined with appropriate linkage analysis methods, the construction of a high-density citrus genome map was carried out. In this study, we have also applied bulked segregant analysis to identify AFLP markers and other marker types tightly linked with *Ctv*, to saturate further the *Ctv* region. A consensus map for the *Ctv* and the nematode resistance regions was constructed by integrating three linkage maps from different populations.

A brief review of literature has been presented on the molecular marker development and the application of AFLP markers in genome mapping. This review also introduced citrus genetics and breeding challenges and the possible impacts of molecular genetic studies on citrus breeding. Genetic linkage analysis programs were introduced briefly. The purpose of this review was to indicate the necessity and desirability of constructing a high-density citrus genome map with AFLP markers. The methodology and the result of these mapping studies are described in the following chapters.

CHAPTER 3
CONSTRUCTION OF A TOTAL CITRUS GENOME MAP BASED ON AFLP MARKERS

Introduction

Citrus is the number one fruit crop and is widely grown throughout tropical and subtropical regions of the world. Long periods of juvenility, large plant size, great heterozygosity, nucellar embryony, and quantitative inheritance of most characters have greatly impeded progress toward genetic improvements in citrus cultivars by conventional breeding methods (Gmitter et al., 1992). The improvement of citrus cultivars by genetic manipulation via molecular tools is becoming possible. Genetic markers have become very efficient and powerful tools in plant breeding (Lande and Thompson, 1990). Genetic maps with a relatively high-density of markers are useful for locating or tagging gene and QTLs of agronomic interest, to provide fundamental information to facilitate marker-assisted breeding, and even to allow map-based cloning of individual genes for plant species with a well-defined genetic map (Leyser et. 1993; Martin et al. 1993). Genetic maps also provide useful clues to understand the biological basis of complex traits and phenomena (Lee 1995). In the perennial crops with long generation times, such as citrus, genetic linkage maps perhaps will provide the greatest potential for increasing the efficiency of cultivar improvement. Citrus genetic maps have been constructed over time since molecular markers have become available. The initial citrus genome maps were based on RFLPs and isozymes (Durham et al., 1992; Jarrell et al., 1992). One of the maps (Durham et al; 1992) was extended by the incorporation of RAPD markers combined with previously mapped RFLPs and isozymes (Cai et al., 1994). The current citrus genome core map consists of nine linkage groups containing 109 RAPD markers, and 51 RFLP and isozyme markers, with a total length of 1192 cM, covering 70-80% of the citrus genome (Cai et al., 1994). Localized genetic linkage maps for horticulturally important traits have been developed in citrus, including maps of the gene for citrus tristeza virus resistance (*Ctv*) (Gmitter et al., 1996; Deng et al., 1997; Fang et al., 1998) gene(s) for citrus nematode resistance (*Tyr1*) (Ling et al., in press), and a gene controlling fruit acidity in citrus (Fang et al, 1998). The QTLs for cold and salt tolerance were also mapped in citrus (Cai et al., 1994; Tozlus et al., in press). However, much of the polymorphisms at the DNA level of citrus are still far beyond recognition; this is especially true when wide intergeneric crosses are involved. There are many genes that remain to be discovered and mapped, including extensive QTLs responsible for various biological functional aspects which will impact citrus cultivar improvements. It is beneficial to have an established framework map well-saturated with molecular markers, for the future mapping of newly identified genes or QTLs, and as a foundation for map-based cloning of important genes.

Recently, amplified fragment length polymorphism (AFLP) markers were developed (Vos et al. 1995) as a new DNA marker system combining the features of RFLPs and PCR. The AFLP marker system involves three steps: (1) digestion of genomic DNA with selected restriction enzyme combinations and subsequent ligation of adapters;

(2) amplification of restriction fragments; and (3) analysis of amplified fragments by denaturing polyacrylamide gel electrophoresis. A great advantage of the AFLP system compared with other marker systems is that it allows simultaneous identification of a large number of amplified products, and therefore it is very widely used currently in genetic mapping of many plant species (van Eck et al. 1995; Mackill et al. 1996; Keim et al. 1997, Qi and Lindhout. 1997; Eujayl et al. 1998, Waugh et al. 1997; Nandi et al. 1997; Wang et al. 1997). The AFLP marker system has been used efficiently in identification of specific genes in segregating populations (Thomas et al. 1995; Cervera et al. 1996), and to identify differentially expressed genes in plants (Bachem et al. 1996; Money et al., 1996). The major advantage of the AFLP markers has been taken to enrich markers in certain regions of genomes (Ballvora et al. 1995; Meksem et al., 1995; Cnops et al., 1996; Simons et al., 1997).

In the present study, we describe the construction of a high-density citrus genetic map using an intergeneric backcross population that was used previously for mapping isozymes, RFLPs, and RAPDs. The marker types involved in this mapping study included AFLPs, RFLPs, RAPDs, SCARs, isozymes, and newly developed RGCs (resistance gene candidates). The previously mapped *Ctv*-linked SCARs, RAPDs and citrus RGC markers (Gmitter et al., 1996; Deng et al. submitted) were integrated in this AFLP genome map. Following are the methods and technical details of developing AFLP markers in citrus, and the construction of the genome map.

Material and Methods

Plant Materials

The intergeneric BC1 population with 71 individuals derived from a cross between *Citrus grandis* ('Thong Dee') and USDA 17-40 (an F1 hybrid of 'Thong Dee' *Citrus grandis* x *Poncirus trifoliata* cv. Pomeroy) was used in this study. This BC1 population was designated the "R family" and was the same population used to construct the RFLP- and isozyme- based citrus genome map (Durham et al., 1992), and later for the RAPD marker-based citrus genome map (Cai et al., 1994). The R family was one of the populations used to identify *Ctv*-linked RAPD markers (Gmitter et al., 1996) and SCAR markers (Deng et al., 1997).

High Quality Genomic DNA Isolation

Young leaves of the parents and hybrids from the R family were used for genomic DNA isolation. Healthy, newly expanded tender leaves were collected from greenhouse grown plants. Leaves were rinsed with deionized water, and used for genome DNA extraction. Isolation of DNA was conducted according to the protocol modified from the standard method (Murray et al., 1980) with slight modifications. The procedures are described in detail, as follows.

1) Approximately 2.0-3.0 g of young leaf material (or tender tips) were ground to a fine power in liquid nitrogen, transferred with liquid nitrogen to a 45ml Oak Ridge centrifuge tube, and placed on ice.

2) Almost immediately, 10 ml of ice cold lysis buffer [1% certyltrimethylammonium bromide (CTAB), 5% polyvinylpyrrolidone (water insoluble PVP), 1.4 M NaCl, 20mM EDTA, 10mM Tris-HCl (pH 8.0), and 350 mM 2-mercaptoethanol] were added to the tube. Tubes were capped and shaken gently to submerge the leaf power in lysis buffer, and incubated on ice 15 min or longer.
3) Tubes were removed from the ice bath, then 10 ml of phenol/chloroform (1:1) [chloroform was a mixture of chloroform/isoamyl alcohol (24:1)] were added and shaken gently to mix well, and placed on ice.
4) Tubes were centrifuged at 25,000 x g, under 4°C, for 15 min. Supernatant was transferred to a set of clean 45 ml Oak Ridge centrifuge tubes, and 10 ml of chloroform were added to each tube. The contents were mixed by gently inverting for 2 min.
5) Tubes were centrifuged at 25,000 x g, under 4°C, for 20min. Supernatant was transferred to a set of clean 45 ml Oak Ridge centrifuge tubes. Then 500 ul of 3M potassium acetate and 2.5 volumes of 95% ethanol were added to each tube, gently inverted a few times, and incubated at -20°C for 60 min or longer.
6) The genomic DNA that condensed and floated on the surface of the potassium/ethanol mixture was transferred to 1.5 ml Ependorf tubes by large transfer pipette, and centrifuged for 10 min at 12,000 x g under 4°C to remove the liquid.
7) DNA pellets were dissolved with 200-500 ul of 1X TE buffer [10 mM Tris-HCl (pH 8.0), 1mM EDTA], and RNase (5ug/ml) was added to each tube according to the solution volume, and treated at 37°C in a water bath for at least 2 hrs.

8) DNA was precipitated again with 95% ethanol and pelleted as described in step 6. The DNA pellets then were washed twice with 95% and 70% ethanol briefly, and dried by air vacuum.
9) DNA pellets were dissolved with 1X TE buffer. The concentration the DNA was determined by analyzing 2 ul samples on a 1% agrose gel and quantitated against a known standar.
10) All the DNA samples were equalized to approximately 100ng/ul, according to gel determination.

Restriction Enzyme Digestion of Genomic DNA

The DNA sample of the parental plants and population individuals were double digested by the restriction enzyme combination of *Eco*RI and *Mse*I (New England Bio-Lab) at the concentration of 2U/ul. For each DNA sample digestion, the digestion mixture had a total volume of 25 ul. The components of the digestion mixture were as follows: approximately 250 ng of the genomic DNA in less of 10 ul, 2 ul of *Eco*RI/*Mse*I (2U/ul), 5ul of 5x reaction buffer [50 mM Tris-HCl (pH 7.5), 50mM Mg-acetate, 250 mM K-acetate], and H_2O to 25 ul. Double digestion was carried out in a 37°C water bath for 3hrs. After the digestion, the mixtures were incubated for 15 min at 70°C to deactivate the enzyme. Completion of the digestion was examined by running 1ul of digestion sample on 1% agrose gel.

Ligation of Adapters

Completed genomic DNA digestions were ligated with *Eco*RI and *Mse*I adapter sequences. The ligation reaction mixture for each sample including 12 ul of genomic DNA digestions, 12 ul adapter ligation solution [*Eco*RI/ *Mse*I adapters, 0.4 mM ATP, 10 mM Tris-HCl (pH 7.5), 10 mM Mg-acetate, 50 mM K-acetate] and 1 ul of T4 DNA ligase [1unit/ul in 10 mM Tris-HCl (pH 7.5), 1 mM DTT, 50 mM KCl, 50% glycerol (v/v)] to make up a total of 25 ul. The ligation reactions were carried out in a water bath at 4°C overnight. The ligation products were diluted ten-fold in 1X TE buffer.

Generation of DNA Templates for Selective Amplification

The DNA templates were generated by pre-amplification. Diluted adapter-ligated genomic DNA fragments were amplified by the *Mse*I primers containing one selective nucleotide (N + 1) and the *Eco*RI primer containing no selective nucleotides (N + 0). Two primers for the pre-amplification reaction were pre-mixed in the ratio of 1:1. Each 21ul pre-amplification reaction consisted of buffer [100 mM Tris-HCl (pH8.3), 15 mM MgCl2, 500 mM KCl] (Life Technologies), 1:10 diluted ligation products, the preamplification primer mixture of *Eco*RI (N+0)/*Mse*I (N+1) (1:1), and 1unit of *Taq* polymerase (Promega). Each reaction mixture was overlaid with mineral oil. Amplification was conducted in a MJ PTC-100 ™ thermal controller with 96 wells (MJ-Research, Inc) under the following conditions: 1 min at 93°C, 1 min at 56°C , and 1 min at 72°C for 20 cycles. Reactions were finally stored in 4°C. After the pre-amplification, reactions were transferred to 600 ul microcentrifuge tubes and 1:100 dilutions were made as follows: transfer 2 ul reaction mixture to a 600 ul microcentrifuge tube containing 198 ul of 1X TE buffer. In addition to generation of DNA template for each individual of the population, a pair of DNA bulks was also constructed based on CTV resistance phenotype results. Diluted pre-amplified DNA template from five CTV resistant individuals and five CTV susceptible individuals were bulked separately. Each individual contributed 20 ul of 1:100 diluted DNA templates, and made up 100 ul bulked DNA templates for each bulks.

Primer Labeling and Primer Combinations

*Eco*RI primers with two or three selective nucleotides were labeled by phosphorylating the 5' end of the *Eco*RI primers with the γ-^{33}P (ATP) and T_4 polynucleotide kinase. The ratio between *Eco*RI primer (27.8ng/ul) and *Mse*I primer (6.7ng/ul) is 1:9 by volume as they constitute the selective primer combination. The total volume of 50-ul labeling reaction consisted of buffer [350 mM Tris-HCl (pH7.6), 50 mM MgCl2, 500 mM KCl, 5 mM 2-mercaptoethanol], 50 uCi of γ-^{33}P (ATP) (2000Ci/mmol) (DuPont), 20U of T_4 polynucleotide kinase (Life Technologies), and 500 ng of *EcoR*I primer. The reaction mixture was mixed gently, briefly centrifuged to collect contents of the tube, and incubated in a water bath at 37°C for 1 hr. The reaction mixture was heated to 70°C for 10 min to inactivate the enzyme, and briefly centrifuged to collect reaction content. The selective primer combination was produced by adding 450ul of *Mse*I primer into the tube, followed by gentle mixing and brief centrifugation.

Selective AFLP Amplification

DNA templates generated by pre-amplification were used for selective amplifications in a total volume of 20 ul comprised of buffer (50 mM KCl, 10 mM Tris-HCl pH 9.0, 1% Triton X-100) (Promega), 2 mM MgCl2, 0.8 mM dNTPs (200 uM of each dNTP), 7.0 ng of *Eco*RI/*Mse*I selective primer combination, 1U of *Taq* polymerase (Promega), and 4 ul of 1:100 diluted preamplified DNA template. Each reaction mixture was overlaid with mineral oil. The same thermal controller was used as the one in the preamplification reaction. Selective amplification was performed with the following cycle profile: one cycle at 93°C for 1 min, 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 1 min; then the annealing temperature was lowered in each of 12 cycles by 0.7°C, which gave a touch-down phase of 13 cycles. The subsequent cycle profile connected with the touch-phase was: 93°C for 30 seconds, 56°C for 1 min, 72°C for 1 min, for 23 cycles. After the amplification, 10 ul of formamid dye (98% formamid, 10 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol) was added to each reaction. Samples then were stored at -20°C.

Gel Analysis

Amplified DNA fragments were analyzed on 6% denaturing polyacrylamide gels. For each gel, 100 ml of 6% acrylamide urea gel solution [acrylamide : N,N'-methylenebioacrylamide (19:1); 7.5 M urea; 5X TBE buffer (500 mM Tris; 500 mM boric acid; 10 mM EDTA (pH 8,0)] were swirl mixed with 500ul of 10% ammonium persulfate and 100 ul of TEMED (N,N,N'N'-tetramethylethylenediamine). Gel solutions were immediately cast into a 40cm (width) x 46 cm (high) glass "sandwich" with 0.4-mm spacers and sharktooth combs (63 wells), and solidified under room temperature for 1 hr or longer. The Bio-Rad 3000 gel electrical electrophoresis system (Bio-Rad) was used for gel analysis. The 1X TBE buffer was used as gel electrophoresis running buffer. The 6% polyacrylamide gel was preheated by running at constant power 55W for 30 min before the samples were loaded. Samples were denatured by heating at 93°C for 5 min on the thermal controller prior to loading to the gel. For each well, 3.5 ul of denatured samples were loaded, and run under constant power 55W for 3 hrs. After the gel cooled, it was transferred to Whatman 2MM paper and vacuum dried for 2 hr at 90°C on a gel dryer (Bio-Rad). The gel then was exposed to autoradiographic film (BioMax MR, Kodak) at room temperature overnight or several days.

Data Scoring and Marker Nomenclature

Bands were visualized on the autoradiographs. Bands present in the pollen parent USDA 17-40, but absent in the seed parent 'Thong Dee', and that appeared to be segregating in the population, were scored as dominant markers. The bands segregating in the population and present in the 'Thong Dee', but absent in 17-40, were scored separately. The number of fragments and polymorphisms generated by each primer pair were summarized to reveal the best primer combinations for the amplification. The unreliable, ambiguous bands were not scored. Because a BC1 population was used in this mapping project, the presence of the AFLP bands implies heterozygosity (Aa) of that locus, and the absence of that for the homozygotes (aa) of the locus. The gel results were visualized and scored twice independently, as presence/absence of a given band. Heterozygotes (Aa) and homozygotes (aa) were designated as “h” and “a” in the linkage analysis file, respectively. Some bands not produced by either of the parents, but which appeared and segregated in the BC1 population were also scored separately. Chi-square tests were performed to check whether individual markers segregated with expected ratios. The current mapping data set consisted of AFLPs, SCARs, RAPDs, and RGCs. AFLP markers were designated according to the name of restriction enzyme primer combinations and the selective nucleotides, followed by the size of amplified fragments. The mapping data of SCARs, RAPDs, and RGCs were obtained from different mapping experiments in the same BC1 population over time (Gmitter et al., 1996; Deng et al., 1997; Deng et al., submitted). The SCAR and RAPD markers were named by beginning with “SC” and “OP” respectively, indicating their primer origin and followed by the fragment sizes. RGC markers were named beginning with “RG” and followed by their origin from the restriction enzyme digestion.

Linkage Analysis

The linkage analysis was based on the currently generated data set, including AFLPs, SCARs, RAPDs, and RGCs.The AFLP marker data set was initially organized by Quattro Pro. computer program. Data of SCARs, RAPDs, and RGCs in this BC1 population were collected from various gel analyses, and most of them were the result of the *Ctv*-linked marker identification. AFLP data were separated by their primer origin and categorized into the three possible genotypes aa x Aa, Aa x aa, and Aa x Aa. The primary AFLP data used in this mapping study were of the aa x Aa type, and they were formatted according to the requirement of JoinMap computer program. Data were analyzed using JoinMap 2.0 (CPRO-DLO). Various ranges of the parameter sets were examined to reach the optimum grouping and ordering of the markers. Markers were first grouped under an array of minimum LOD score from 3.0 to 7.0. Finally, all the markers were grouped under the minimum LOD threshold value of 5.8 and the maximum recombination value of 0.49. Then, all the markers were split into different groups by the data file splitting module according to the grouping result. For each linkage group, the recombination estimation module was used to calculate the recombination frequency values for each group of markers pairwise under the LOD threshold value. The least restrictive LOD and recombination threshold value were used to ensure that all the pairwise markers in a linkage group could be used in the subsequent mapping command. The marker sequence ordering within each linkage group was then performed by the map construction module with an LOD score of 0.01; the “Jump” (Stam and van Ooijen 1995) threshold value varied between 3.0 to 5.0 for different linkage groups depending on the size of that linkage group. The goodness-of-fit was measured for each marker added on to the core linkage group, and it was determined by the “Jump” value. Such determinations were performed for three rounds by the map construction module,with the first and the second round determinations under the same strength of “Jump” value.“Fixed sequences” were established from the second round determination, and used for the third round of map calculation and marker order placement without the “Jump” restriction. The orders of the marker sequence in a certain genomic region were determined by the “ripple” (3.0) command for every three adjacent markers in that region. By setting the 'triplet' threshold to a very stringent level (6.0), only one top inferred fixed order linkage group was generated and used as the linkage map drawing file. The Kosambi map function was used in all linkage analyses.

Results

Genomic DNA Isolation

The concentration of genomic DNA isolated from all the individuals varied from 50ng/ul to 1500ng/ul, depending on the leaf material available at the time. For AFLP analysis, 250ng DNA with good purity in a solution less than 18 ul is sufficient for restriction enzyme digestion. The purity of the DNA was determined by spectrophotometry as the ratio of the optical density reading between 260 and 280 nm; the lowest ratio was 1.68, but most of the samples were greater than this value, reaching 1.8 or higher. The purity of the DNA from each individual was suitable for the AFLP analysis, and was confirmed by the complete enzyme digestions in the AFLP template preparation.

AFLP Analysis

Twenty six *Eco*RI+2 /*Mse*I+3 nucleotide primer combinations, and thirteen *Eoc*RI+3 /*Mse*I+3 nucleotide primer combinations (total of 38 primer combinations) were tested against the two parents, resulting in 2516 amplified bands with an average of 68 amplified bands per primer pair. All of the primer combinations produced polymorphic fragments between the parents. The *Ctv*-linked AFLP fragments were visualized and confirmed by the correspondent polymorphism between DNA bulks (CTV resistant and susceptible bulks) and the parents (Figure3-1AB). From the 38 primer combinations, a total of 505 polymorphic bands generated against two parents (20% of the total amplification products) were scored. The scoreable markers ranged in size from 50-1280bp, but most of the markers ranged from 100-850bp. On average, 12.6 AFLP markers were produced by each primer combination (Table3-1). From the 505 polymorphic bands, 390 AFLP markers (77.2%) present only in the pollen parent 17-40 but absent in the recurrent parent 'Thong Dee' were selected and used for map construction. Differences in clarity between primers *Eco*RI +2 and *Eco*RI +3 were observed. The *Eco*RI +3/*Mse*I +3 primer combination amplified more clear bands with much less background than the *Eco*RI +2/*Mse*I +3 primer combinations, in genenal. The numbers of polymorphic bands generated by each different primer combination varied from 3-29 bands. The average number of scored markers for the *Eco*RI +3/*Mse*I +3 primers was 9.6, in comparison with 12.3 for the *Eco*RI +2/*Mse*I +3 primers. Finally, a total of 371 markers including 344 AFLPs, and 26 RAPDs, SCARs and RGCs markers, and *Ctv*, were used to construct the linkage map.

Map Construction

Under a relatively high LOD threshold (5.8), a total of 318 AFLPs (86% of the total AFLPs), all the 26 RAPDs, SCARs and RGCs, and *Ctv* (total of 345 markers) were grouped successfully into 11 major linkage groups. There were 26 AFLP makers that formed 12 minor linkage groups; among these 12 groups, 10 consisted only two AFLP markers, and the other 2 consisted only of three AFLP markers. These 12 minor linkage groups were derived from the process of linkage group formation by gradually increasing the minimum LOD threshold value (from 3.0 to 5.8, in 0.2 increments). Twenty six AFLPs could not be grouped together within a confident LOD threshold level, and they were not assigned to any linkage group.

For each linkage group, the linkage significance between each marker was confirmed by the result of subsequent map construction calculation. By giving a "jump" value (3.0 to 5.0), the increased chi-square value due to the "add on" marker was limited in an acceptable range, and all the markers were eventually placed on to the core linkage group with significant confidence. In the map construction process, there was not a fixed marker order supplied by the author, but "fixed marker sequences" were generated for each linkage group by the second round of "Jump" determination. The "fixed marker sequences" then served as the core map order for the ultimate marker placement in each linkage group. The 11 major linkage groups ranged from 29.8 cM to 148.9 cM in length, and the groups carried 4 to 94 markers with an average of 31 markers per linkage group (Table 3-2). The clustering of the markers in certain linkage groups, as well as in certain regions of the linkage group, was observed (Figure 3-2). Among 11 major linkage groups, only six intervals between two adjacent markers were in excess of 10 cM, and the largest interval between two markers was 18.4 cM in the linkage group III. The map has a total length of 858.6 cM, with average marker interval distance of 2.7 cM, which was less than the RAPD-based citrus map (Cai et al., 1994).

The markers generated previously that were linked to *Ctv* were all mapped onto the linkage group I, including RAPDs and SCARs. $SCB11_{700}$, $SCAM02_{1000}$ and $SCAD08_{1100}$ (Deng et al. 1997), and RAPD marker $OPC19_{1000}$ co-segregated with *Ctv* in this backcross family. $SC07_{650}$ was mapped 1.4 cM away from *Ctv*; this marker was tightly associated with a major citrus nematode resistance QTL region (*Tyr*1) in another intergeneric backcross population (Ling et al; in press). Four newly developed RGC markers (RGPt9Hinf, RG18P33, RG11R11 and RGPt8TaqI) were mapped also in linkage group I. RG18p33 and RG11R11 also co-segregated with *Ctv*. RGPt8TaqI was closely associated with *Tyr*1 and mapped on the other side of $SC07_{650}$ (Ling et al; submitted). Three AFLP markers ETTMCTA175, EAGMCAC350 and EACTMCAA260 were identified that co-segregated with *Ctv*.

Marker Segregation

A total of 187 (39%) of the 479 AFLP markers, showed segregation patterns skewed from a 1:1 ratio at $P = 0.05$. Among the skewed AFLP markers, 62 were skewed toward the donor parent 17-40, and 125 were skewed toward the recurrent parent ‘Thong Dee’. *Ctv* also was skewed from the 1:1 ratio, toward the recurrent parent ‘Thong Dee’. All of the AFLP markers closely linked to *Ctv* displayed skewed segregation, as *Ctv* itself. Previously identified *Ctv*-linked RAPDs and SCARs also showed varying degrees of skewed segregation (Gmitter et al., 1994; Deng et al., 1997). All of the *Ctv*-linked markers (RAPDs, SCARs and RGCs) clustered in the linkage group I. Those AFLP markers that were skewed toward the pollen parent 17-40 were distributed toward to the distal region of linkage groups II, III, IV, VII, VIII, and IX. On the other hand, the AFLP markers skewed towards the recurrent parent 'Thong Dee' were rather evenly distributed in most of the linkage groups, except linkage group VIII and IX in which skewed markers were found in the distal regions. The skewed markers mapped close to each other and formed clustered groups, with the number of markers varying from 2 to 10. Linkage group VII had 10 skewed makers clustered together at the end, which covered 21% of that linkage group. The percentages of skewed markers in each linkage group is indicated in Table 3-2.

Discussion

Genetic Variation Detected by AFLPs in *Citrus*

Polymorphism in *Citrus* at the DNA level has been reported with different molecular markers systems. Durham et al. (1992) reported that 14 out of 20 citrus genomic library probes tested generated 58 useful RFLPs in an intergeneric BC1 population of *Citrus* and *Poncirus*. The high level of polymorphism was found also between *Citrus* and *Poncirus*, by Jarrell et al. (1992). In that study, 37 RFLPs were generated from 35 cDNA and *Pst*I genomic library clones and along with other isozyme markers, were used to construct a linkage map with an F_2 type intergeneric population (Jarrell et al., 1992). Cai et al. (1994) used the same BC1 population as Durham et al.

(1992) and constructed a RAPD-based map. However, the highest number of polymorphic bands generated by a single RAPD primer was 11. Microsatellite markers have also been used to evaluate polymorphism in *Citrus*. Kijas et al. (1997) tested 14 microsatellites with the mapping population reported previously (Jarrell et al., 1992), and found 7 that revealed polymorphism; these markers were integrated into the linkage map constructed by Jarrell et al. (1992). The result from previous citrus mapping studies (Durham et al., 1992; Jarrell et al., 1992) have demonstrated a lower percentage of aberrant segregation when compared with the intergeneric crosses of other organisms. This suggests that *Citrus* and *Poncirus* have retained a large amount of structural and functional homologies, although these two genera are taxonomically distinct at the morphological level (Jarrell et al., 1992). Although these various marker systems reveal substantial levels of polymorphism, there still are a large proportion that either somehow fail to reveal segregating polymorphisms in the *Citrus/Poncirus* intergeneric F1 backcross or F_2 progenies, or produce patterns too complicated to analyze (Durham et a., 1992; Jarrell et al., 1992; Cai et al., 1994; Kijas et al., 1997). Therefore, there is potential for discoverying polymorphisms by using more effective molecular marker system to search the genome in a more complete fashion, and AFLP is one of the most effective new marker systems available at this time.

The AFLP system used to detect the new markers in *Citrus* has combined both four- and six-base restriction enzymes. An average of 12.6 scoreable polymorphic markers were generated per primer pair between parents in this study. This number is greater than an average of 3.9 in melon (Wang et al., 1997) and 7.5 in rice (Nandi et al., 1997), but lower than 18 in soybean (Maughan et al., 1996) and 26 in sorghum (Boivin et al., 1999); this is expected because the genome complexity of soybean and sorghum is much higher than that of citrus. It could be useful to try other four-base cutters in combination with different six-base cutters to explore higher levels of polymorphism in the citrus genome, thus to extend more complete genome coverage. From our mapping result, the relative clustering of markers in the certain linkage groups also imply the necessity of such attempts. It has been reported in the AFLP mapping study of soybean, that using additional primer pairs with a six-base-cutting restriction enzyme such as *Pst*I resulted in different distribution patterns of the AFLPs in the soybean genome (Young et al., 1998). Using enzyme systems other than *Eco*RI/*Mse*I may result in more evenly distributed markers in some linkage groups.

It is known that in plant genomes, there are hypo-methylated sequences associated with actively expressed genes, while the hyper-methylated sequences are considered transcriptionally inactive. In our study, AFLP markers were generated by using a methylation insensitive restriction enzyme, *EcoR*I (non-sensitive to CG). Thus it is very possible that some particular regions of the citrus genome, such as heterochromatin or centromeres and telomeres, can be accessible to *Eco*RI, and AFLPs can be identifed there. In these regions, crossing-over during meiosis is greatly reduced (Luckaszewski and Curtis 1993), and markers tend to be clustered. *Eco*RI-based AFLP markers mapped on the citrus genome should include both coding and non-coding regions, while using a methylation sensitive restriction enzyme such as *Pst*I should produce AFLP markers more likely in the coding regions. A similar logic was suggested by Cai et al. (1994) in citrus genetic mapping and by Boivin et al. (1999) in sorghum genetic mapping when *Pst*I-derived genomic RFLP probes were used. The genetic mapping study of *Lens* sp. (Eujayl et al., 1998) showed much greater polymorphism detection by the *Pst*I/*Mse*I primer pair (70%) than *Eco*RI/*Mse*I primer pair (15%). Whether or not *Pst*I-based AFLP markers would produce similar results in citrus remains to be determined.

Regardless of this, the polymorphic markers obtained in the present study were sufficient to construct a relatively high density linkage map of citrus. In this study, we have used *Eco*RI selective nucleotides of all the A-and T- combinations, with different *Mse*I selective nucleotides of all the C-- combinations; however, not all combinations of the two primers were tested, only 33 out of 64 *Eco*RI +2/*Mse*I +3 possible primer combinations were randomly used (Table 3-1). Five combinations of *Eco*RI +3/*Mse*I +3 primer pairs EACA/MCAG, EACC/MCAA, ECAA/MCAC, EACT/MCAA, and EAGG/MCTT were tested randomly, and all of them amplified clear and easily scored markers (Figure 3-1B). This may be due to increasing the specificity for primer matching by adding one more selective nucleotide to the *Eco*RI primer. Theoretically the *Eco*RI+2/*Mse*I+3 type primer pairs should amplify more products than the *Eco*RI+3/*Mse*I+3 type primer pairs. Thus it is interesting that the primer pair EACT/MCAA amplified 15 scoreable markers, which is greater than the 14 scoreable markers generated by EAC/MCAA; this number is also greater than the average number of markers amplified by *Eco*RI +2/*Mse*I +3 primer pairs (12). It is clear that the use of only the *Eco*RI/*Mse*I enzyme system with different combinations of selective nucleotides would, in principle, provide sufficient markers for high-density mapping of citrus. The potential to improve this system could be realized by applying more enzyme combinations, according to the functional characteristics of the enzymes.

Mapping Population

Many plant genetic linkage maps have been generated using either F_2 or backcross populations. In citrus, the first two genetic maps were constructed mainly with RFLP markers and isozymes (Jarrell et al., 1992; Durham et al., 1992), using F_2 and F_1 backcross populations. In both cases, the intergeneric crosses between *Citrus* and *Poncirus* were involved. For plants like citrus, with a broad range of related genera but a fairly narrow intraspecific gene pool, more segregating polymorphisms are expected to be found in intergeneric than in interspecific populations. For the dominant type marker system such as RAPD and AFLP, backcross populations are more suitable than F_2 populations although only one (rather than two) recombinant gametes are sampled in each plant (Reiter et al., 1992).

Markers with Skewed Segregation

The percentage of skewed markers in this study was 39%. This result is similar to that of others citrus genetic mapping studies. Segregation distortion of RFLPs and isozymes was reported as 37% in the *Citrus* x *Poncirus* backcross population, while 29% of the markers in an interspecific *Citrus* cross had distorted ratios (Durham et al., 1992). It was reported that 40% of the RAPD markers displayed skewed segregation patterns when RAPD markers were mapped in the same backcross population (Cai et al., 1994).

In another citrus genetic mapping study, 24% of RFLP markers showed distorted segregation patterns when using an F_2 intergeneric population (Jarrell et al., 1992). From a comprehensive view, the degree of the distortion seems independent from the marker system used, but rather it is related to the population structures. Distorted segregation between alleles has been discussed widely and has been commonly observed in both intra-and inter-specific crosses of plant species other than *Citrus*. AFLP analysis of a rice intraspecific population found that 33% of the AFLP markers deviated from the expected ratio (Nandi et al., 1997). In peach, genetic study with AFLP markers using an interspecific F2 population revealed that 12.7% AFLP markers showed distorted segregation (Lu et al., 1998). The level of segregation distortion in *Citrus* is not too high when compared with other annual or perennial species (Helentijarise et al., 1986; Landry et al., 1987; Gebhardt et al., 1989; Pillen et al., 1992; Eujayl et al., 1998; Lu et al., 1998). All DNA markers systems that have been used in *Citrus* genetic studies have exhibited various degree of distorted segregation (Durham et al., 1992; Jarrell et al., 1992; Cai et al., 1994; Gmitter et al., 1997; Deng et al., 1997), which indicates that not all DNA marker loci are neutral and both coding and non-coding sequences can be under selection pressure (Landry et al., 1992). One possible explanation for distorted segregation in this study, could be linkages to incompatibility loci (Wricke and Wehling 1985) since incompatibility exists in *Citrus*. Another possible explanation could be linkage to the lethal alleles in gametes (Pillen et al., 1992).

In our study, 67% of the total skewed AFLP markers were toward to the recurrent parent 'Thong Dee', while 33% were skewed toward to the donor parent 17-40. Results from Cai et al. (1992) showed that almost all of the distorted loci were toward to the recurrent parent. Similar results were also found in number of other studies (Durham et al., 1992; Jarrell et al., 1992). On the AFLP citrus map, the clustering of the distorted loci was very obviously displayed in specific linkage groups. The presence of these large blocks of loci skewed toward one parent or another may have implicated the existing QTLs in that certain genomic region (Cai et al., 1994). The distribution of clustered segregation-distorted markers also suggests those loci are likely linked to the genes directly exposed to selection.

Map Construction

A genetic linkage map of *Citrus* was constructed by JoinMap with 336 DNA markers (310 AFLPs, 16 RAPDs, 6 SCARs and 4 RGCs) and 1 phenotypic marker (*Ctv*) covering 858.6 cM of the *Citrus* genome with 11 linkage groups, with the average distance 2.7 cM between markers. A previous map from the same family (Cai et al., 1994) had 9 linkage groups generated by MapMaker, spanning 1192 cM with an average distance of 7.5 cM between loci. The marker density of this genetic map was significantly increased. The unequal number of the linkage groups obtained from these two different mapping experiments may reflect the uneven distribution of the RAPD priming sites and the enzyme access sites of *Eco*RI/*Mse*I in the genome. The difference in length between the previous map and the current AFLP map demonstrates a reduction in map distances calculated by JoinMap in comparison with MapMaker; similar results were reported also by Cai et al. (1992) and Kijas et al. (1997). The calculation differences between these two computer mapping programs may have contributed also to the unequal number of linkage groups in these two mapping studies. More segregation-distorted markers were used in this map construction than in previous maps; this might be another reason for map length differences. The same suggestion was made by Cloutier et al. (1997), when analysing the RFLP mapping inaccuracy in *Brassica napus* L.

Although the previous linkage map was condensed to reach the haploid chromosome number (n = 9), there were 14 linkage gaps larger than 20 cM distributed across 8 linkage groups, and the largest one being 29.9 cM in linkage group I. In the AFLP map, in spite of 11 linkage groups and shorter map coverage (858.6 cM), there are only 6 linkage gaps. These gaps are less than 19 cM and all of them exist at the end of linkage groups (the largest gap was 18.4 cM in linkage group VII). The genome of *Citrus* has been estimated to span from 1500 cM to 1700 cM (Jarrell et al., 1992), thus it is clear that neither of these two *Citrus* genome maps has reached saturation by markers. In both mapping studies, insufficient numbers of markers may be the cause of shorter genome coverage. A similar conclusion was suggested in the mapping study of *Phaseolus vulgaris* L. with RFLP markers (Vallejos et al., 1992). Shorter genome coverage could also be the consequence of the fairly small size of populations used in these studies; perhaps some key recombinants were not identified, and thus some gaps remain to be filled. It has been suggested that increasing population size, and not the number of markers, is more likely reduce the number of linkage groups (Kesseli et al., 1994; Keim et al., 1997; Wang et al., 1997).

Generally, with the progeny number less than one hundred, it is difficult to reach high resolution of the map overall, but the relative position of pre-identified genes or known QTLs regions can be more accurately determined. It will be more constructive to apply AFLP techniques to larger populations for more complete total genome map construction. Furthermore, the AFLP markers can be converted to either PCR primers or RFLP probes to facilitate integration with other linkage maps (Baudracco-Arnas and Pitrat 1996), and used for fingerprinting of YAC and BAC libraries (Simons et al., 1997; Zhang and Wing 1997). At this point, it is necessary to integrate all AFLP markers with all previously mapped markers to construct a much more complete citrus genome map.

The SCAR, RAPD, and RGC markers derived from various mapping experiments aimed to clone the citrus tristeza virus resistant gene (*Ctv*) were all mapped in linkage group I. Some of the above markers have been tested in the current population, and in another family with nearly 700 individuals, or with the recombinant group (with 101 individuals) of this larger family. All of the AFLP markers located in the linkage group I were integrated into a localized linkage map based on this larger family, and a high-density consensus map for the *Ctv* gene region was constructed (See Chapter 5).

In the course of map construction there were 12 minor linkage groups generated by 26 AFLP markers. The JoinMap computer program determined that these markers do not have significant linkage with at least 2 other loci, which led to failure of the map distance calculation. This would be the consequence of insufficient number of markers for the linkage establishment or to join them with the major groups. There were 89 AFLP markers also of the Aa x aa or Aa x Aa genotype, not included in this map. It is possible to construct a genetic linkage map for *Citrus grandis* based on these AFLP data. This attempt could be accomplished when the same genotype of the RAPDs, RFLPs, and isozymes from previous mapping studies were joined with the AFLP markers.

Conclusion

AFLP is a rapidly emerging, genetic molecular marker technique which is gaining widespread application in many areas of genetic studies, including genetic linkage mapping and gene cloning through a genome-based approach. It has been shown to be both reliable and reproducible in many organisms, including plants (Folkertsma et al., 1996; Mueller et al., 1996; Huys et al., 1996; Lin et al., 1996; Cervera et al., 1996; Waugh et al., 1997; Mackill et a., 1996; Boivin et al., 1999). In addition, it does not require any DNA sequence information from the organism prior to apply, and it is information rich due to its high multiplex ratio (Powell et al., 1996; Russell et al., 1997; Milbourne et al., 1997). By applying the AFLP technique to citrus genetic mapping, we were able to address the AFLP profiles in the *Citrus* and *Poncirus* genomes. The AFLP marker's amplification ability, the polymorphic levels presented in the intergeneric citrus backcross population, and the potential use as a reliable molecular marker for high resolution mapping of *Citrus* were discussed.

The development of AFLP markers in citrus was fully explored, and it is the first to demonstrate the potential of using AFLP markers for genetic mapping. The AFLP stability, reproducibility, and efficiency were confirmed further in this study. From the result of the study, AFLPs were able to generate a higher density map, and yield higher resolution, than previous citrus maps. It has been demonstrated also that AFLP markers are reliable and efficient for linkage mapping, even when only small populations are available. Based on the information provided on the linkage group I, the order of the pre-determined RAPD and SCAR loci remained unchanged, although the entire linkage group was highly populated with many AFLP markers. It is obvious that the citrus genome has not been saturated with markers, since the number of linkage groups generated is greater than the haploid chromosomes number of citrus ($n = 9$), and the total map length is less than the estimated *Citrus* genome length. It is likely that the minor linkage groups will join with larger linkage groups with analysis of additional markers. Like RAPD markers, many identified polymorphic AFLP bands were subsequently excluded from segregation and linkage analysis because of poor resolution or instability. Therefore, making the best effort technically to improve the gel resolution is very critical and necessary, and AFLP markers must be carefully evaluated prior to use for mapping or other purposes.

In this experiment, we have only used 40% of the possible primer combinations available, so there is still large potential for marker discovery. It will be necessary in the future to screen more primer combinations, including utilization of different restriction enzyme systems. AFLP markers used in the map construction were all manually scored as dominant type markers, but it is possible that some co-dominant type markers can be scored with suitable gel scanning methods and software programs to provide more information.

This study has demonstrated that AFLP markers are feasible for genetic mapping, and showed great ability of densifying a *Citrus* genome map made with a relatively small population. The AFLP system should be very helpful in searching for specific gene regions and QTLs by increasing the marker density in an accelerated fashion. It is our goal to construct a high-density citrus map to identify genes that are horticulturally important, such as *Ctv*, genes for tolerance to Phytophthora, for resistance to citrus nematodes (*Tyr*1), and QTLs for cold hardiness, salt tolerance, tree vigor, fruit size, quality, etc. The donor parent of this backcross population is *P. trifoliata* which possesses many of the known disease and pest resistant genes in citrus, and it is extremely cold-hardy. AFLP markers identified closely linked to those important genes and QTLs can be converted to SCAR markers to facilitate marker-assisted-selection in a single and inexpensive PCR assay. Those selected AFLPs will be evaluated in a larger recombinant group to clarify their genetic distance to *Ctv*.

Table 3-1 AFLP primer combinations tested and the number of scoreable markers amplified from USDA 17-40 in each pair used for map construction. The top row contains *Mse*I primers with 3 selective nucleotides shown, and the left column has *Eco*RI primers with 2 and 3 selective nucleotides. The average and total number of scoreable markers for each pairwise primer were indicated. ND- primer pairs not used in this mapping study.

Primer	M-CAA	M-CAC	M-CAG	M-CAT	M-CTA	M-CTC	M-CTG	M-CTT	Average	Total
E-AA	8	ND	ND	16	ND	ND	12	12	12	48
E-AC	14	10	ND	ND	10	10	6	ND	10	50
E-AG	19	6	13	ND	13	ND	ND	8	11.8	59
E-AT	ND	ND	ND	11	ND	12	ND	24	15.7	47
E-TA	10	ND	9	17	14	ND	8	12	11.7	70
E-TC	ND	5	ND	11	7	ND	ND	ND	7.6	23
E-TG	ND	ND	6	14	ND	8	7	14	9.8	49
E-TT	ND	ND	ND	ND	29	16	13	14	18	72
E-ACA	ND	ND	9	ND	ND	ND	ND	13	11	22
E-ACC	6	12	ND	ND	ND	ND	ND	ND	9	18
E-ACT	15	ND	ND	11	ND	7	ND	ND	11	33
E-AGG	ND	11	ND	ND	ND	ND	ND	3	7	14
Average	12	9.5	9.3	13.3	14.6	11.5	9.2	12.4		
Total	72	38	37	80	73	46	46	87		**505**

Table3-2 Marker distribution among the linkage groups of AFLP-based citrus genome map.

Linkage groups	Number of markers [a]	Length (cM)	Marker interval (cM)[b]	Number of skewed markers [c]
I	67	91.5	2.2	19(28%)
II	47	109.1	2.3	11(23%)
III	94	148.6	1.6	21(22%)
IV	30	107.8	3.6	2 (7%)
V	24	106	3.4	9 (38%)
VI	26	88.8	4.4	11(42%)
VII	23	67.0	2.9	18 (78.2%)
VIII	9	39.6	4.4	1(11.1%)
IX	8	39.5	4.9	5 (63%)
X	5	30.9	6.2	2 (40%)
XI	4	29.8	7.5	2 (50%)
Minor group	26	NA	NA	9 (35%)
Total	363	858.6	2.5	110 (30%)

[a] Markers including AFLPs, RAPDs, SCARs, and RGCs.

[b] Average genetic distance between markers.

[c] Based on *P* value of 0.05. In parentheses is the percentage of skewed markers in each linkage group.

Figure 3-1(AB): Autoradiograms obtained with two primer pair combinations, showing segregation of AFLP markers alleles. The parental DNA samples and two DNA bulks based on CTV resistance and phenotype were listed in the first 4 lanes, followed by some individuals of the population. P1 = USDA 17-40 and P2 = 'Thong Dee' pummelo; R = CTV resistance bulk, S = CTV susceptible bulk. (A) AFLP markers produced with primer combination ETGMCTT. (B) AFLP markers produced with primer combination EACTMCTC. AFLP fragment size was indicated by ^{33}P-labeled DNA size marker. The AFLP markers used for mapping are indicated by *arrow*.

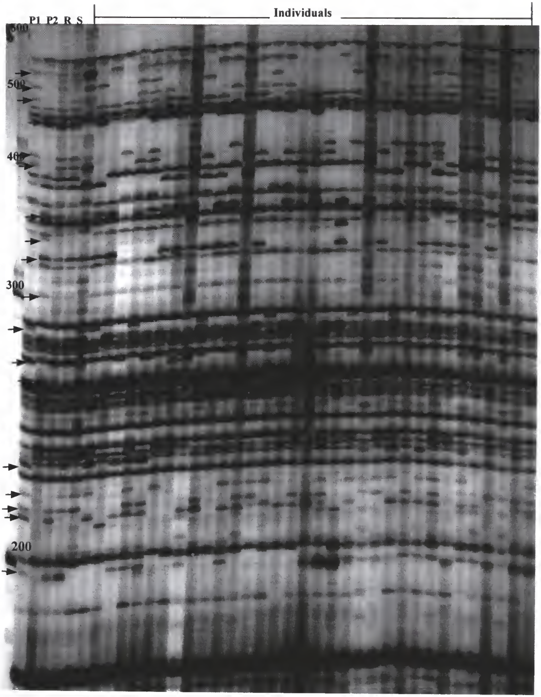


Figure 3-1 A

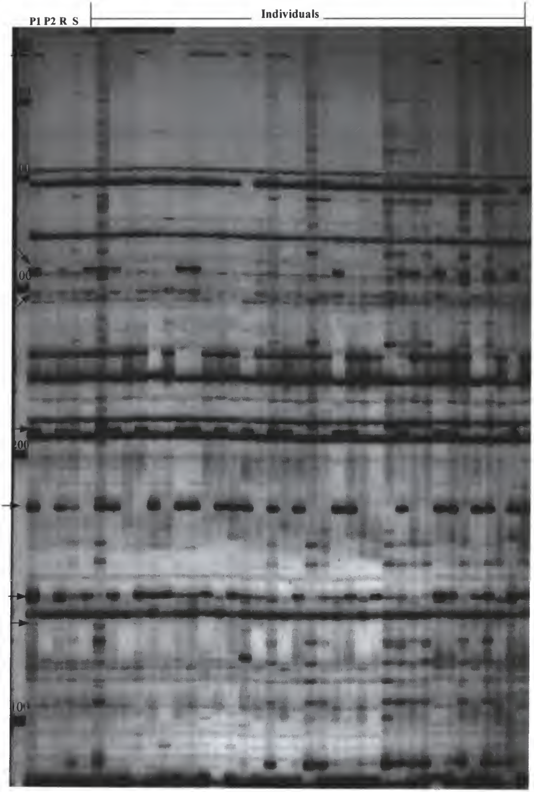


Figure 3-1 B

Figure 3-2: AFLP marker-based citrus genetic linkage map. Markers linked with *Ctv* were consolidated on linkage group I. The markers with designations of *EcoR*I/*Mse*I primers are AFLP markers. RAPDs, SCARs, and RGCs are designated with “OP”, “SC” and “RG” respectively. The recombination values listed on the left side of the linkage core line were calculated by JoinMap (Stam and van Ooijen 1995) using the Kosambi function. Markers listed on the right side of the linkage core line are all of the aa x Aa type. Loci displaying distorted segregation toward to recurrent parent ‘Thong Dee’ are indicated by single *asterisks* “*”, and loci distorted toward to donor parent USDA17-40 are marked with double *asterisks* “**”.

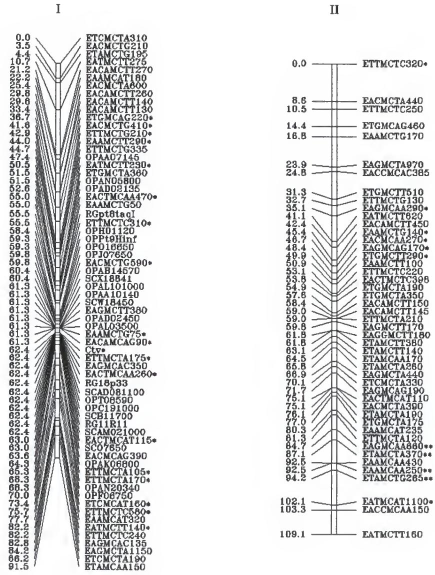
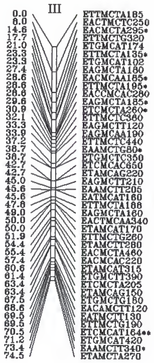
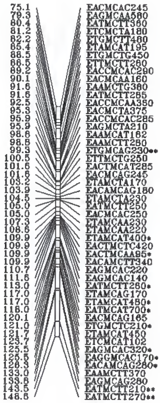
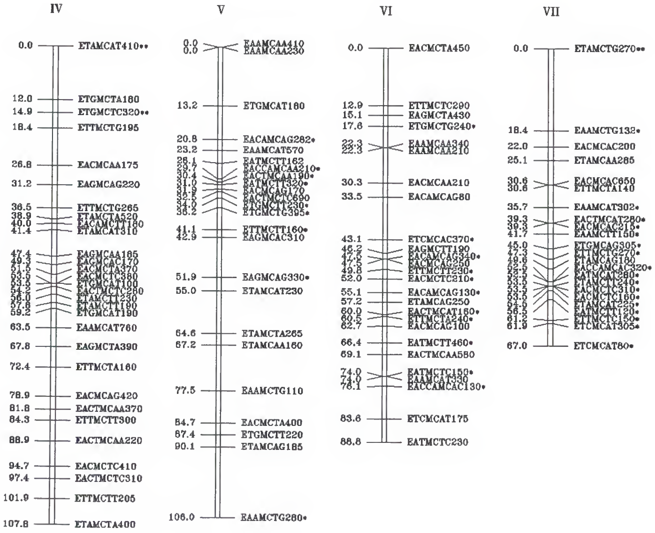

I
0.0 ETCMCTA310
3.5 EACMCTG210
4.4 ETAMCTG195
10.7 EATMCTT275
21.2 EACAMCTT270
22.2 EAAMCAT180
25.4 EACMCTA600
29.8 EACAMCTT260
29.8 EACAMCTT140
33.4 EACAMCTT130
36.7 ETGMCAG220*
41.6 EACMCTG410*
42.9 ETTMCTG210*
44.0 EAAMCTT290*
44.7 ETTMCTG335
47.4 OPAA07145
50.5 EATMCTT230*
51.5 ETGMCTA360
51.5 OPAN05800
52.6 OPAD02135
55.0 EACTMCAA470*
55.0 EAAMCTG50
55.5 RGpt8taqI
55.5 ETTMCTC310*
58.4 OPH01120
59.3 OPPt9Hinf
59.3 OPO16650
59.8 OPJ07650
59.8 EACMCTG590*
60.4 OPAB14570
60.4 SCX18841
61.3 OPAL101000
61.3 OPAA10140
61.3 SCW18450
61.3 EAGMCTT380
61.3 OPAD02450
61.3 OPAL03500
61.3 EAAMCTG75*
61.3 EACAMCAG90*
62.4 Ctv*
62.4 ETTMCTA175*
62.4 EAGMCAC350
62.4 EACTMCAA260*
62.4 RG18p33
62.4 SCAD081100
62.4 OPT08590
62.4 OPC191000
62.4 SCB11700
62.4 RG11R11
62.4 SCAM021000
63.0 EACTMCAT115*
63.0 SC07650
63.6 EACMCAG390
64.3 OPAK06800
65.3 ETTMCTA105*
68.3 ETTMCTA170*
68.3 OPAN20340
70.0 OPF06750
73.4 ETCMCAT160*
75.7 ETTMCTC580*
77.7 EAAMCAT320
82.2 EATMCTT140*
82.2 ETTMCTC240
82.8 EAGMCAC135
84.2 EAGMCTA1150
86.2 ETCMCTA190
91.5 ETAMCAA150
II
0.0 ETTMCTC320*
8.6 EACMCTA440
10.5 ETTMCTC250
14.4 ETGMCAG460
16.8 EAAMCTG170
23.9 EAGMCTA970
24.8 EACCMCAC385
31.3 ETGMCTT510
32.7 ETTMCTG130
35.1 EAGMCAA290*
41.1 EATMCTT620
42.4 EACAMCTT450
45.4 EAAMCTG140*
46.7 EACMCAA270*
48.4 EAGMCAG170*
49.9 ETGMCTT290*
50.9 EAAMCTT100
53.1 ETTMCTC220
53.8 EACTMCTC398
54.9 ETGMCTA190
57.6 ETGMCTA350
58.4 EACAMCTT150
59.0 EACAMCTT145
59.0 ETTMCTA210
59.8 EAGMCTT170
61.8 EAGGMCTT180
61.8 ETAMCTT380
63.1 ETAMCTT140
64.5 ETAMCAA170
65.8 ETAMCTA280
66.9 EAGMCTA440
70.1 ETCMCTA330
71.7 EAGMCAG190
75.1 EACTMCAT110
75.1 EACMCTA390
76.1 ETAMCTA190
77.0 ETGMCTA175
80.3 EAAMCAT235
81.3 ETTMCTA120
84.7 EAGMCAA660**
87.1 ETAMCTA370**
92.5 EAAMCAA430
92.5 EAAMCAA250**
94.2 ETAMCTG265**
102.1 EATMCAT1100*
103.3 EACCMCAA150
109.1 EATMCTT160

Figure 3-2

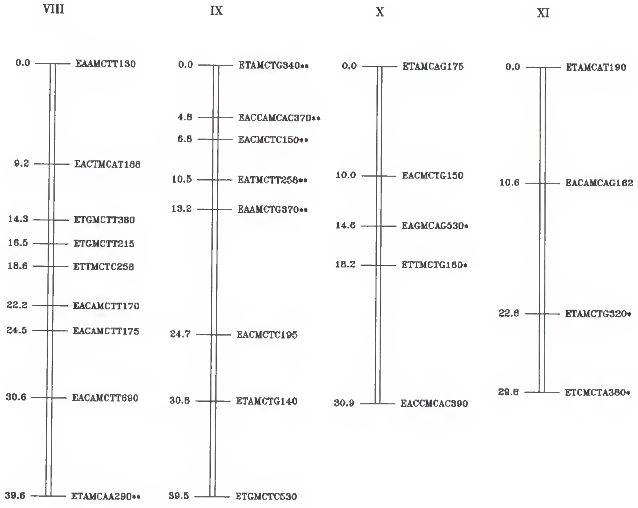




(Figure 3-2 continued)



(Figure 3-2 continued)



(Figure 3-2 continued)

CHAPTER 4

INTEGRATION OF CITRUS GENETIC MAPS WITH DIFFERENT MOLECULAR MARKERS AND QTL ANALYSIS

Introduction

In recent years, DNA markers have become essential tools for plant breeding (Tanksley et al., 1989; Gebhardt and Salamini 1992; Rafalski and Tingey 1993; Kurata et al., 1994; Tanksley et al., 1992; Bell and Ecker 1994). RFLP, RAPD, and AFLP are the three main kinds of DNA markers that are most frequently used in plants. They differ in their principles, applications, polymorphism rates, and cost and time requirements. RFLPs are particularly well suited for the construction of linkage maps and allow synteny studies because of their locus specificity and co-dominant nature. For example, the use of RFLP clones has revealed that chromosome segments of rice and maize (Ahn and Tanksley 1993), and of rice and wheat (Kurata et al., 1994) contain DNA markers in a very similar order. The RAPDs have been particularly well adapted to efficient, non-radioactive DNA fingerprinting of plant genotypes for intra- and inter-specific genetic relationships (Welsh and McClelland 1990; Thormann et al., 1994), as well as the mapping studies (Baudracco-Arnas and Pitrat 1996; Torres et al., 1993; Warburton et al., 1996) including citrus (Cai et al., 1994, Gmitter et al., 1996). AFLP, a new technology developed by Zabeau and Vos (1993), combines the strengths of different marker systems and provides new opportunities for mapping in plants and other organisms. AFLP is well suited for species that have a relatively narrow gene pool and relative low levels of RFLP and RAPD polymorphism; citrus is one such species. The AFLP marker system has a greater ability to provide large numbers of polymorphisms in a single run than other marker types (descried in Chapter 3).

Genetic studies using AFLP markers or combined with other type of markers have been adapted widely in many plant species and other organisms (Becker et al., 1995; Wang et al., 1997; Janssen et al., 1996; Liu et al., 1998). The rapid generation of a large number of reproducible markers by AFLP has also enabled researchers to fill remaining genetic gaps in the maps created with other markers, and to produce high density genetic maps (Boivin et al., 1999).

Genetic linkage maps of molecular markers offer a powerful tool to investigate the genetic architecture of polygenic traits and potentially to assist in their manipulation through marker-assisted selection and breeding. In recent years, a numbers of studies were conducted using molecular markers to investigate the inheritance of quantitative traits in annual crops (Stuber et al., 1992; Dudley 1993; deVicente and Tanksley 1993; Hombergen and Bachmann 1995; Lin et al., 1995; Paran et al., 1996; Redoña and Mackill 1996; Kreike et al; 1996; Zhu et al., 1999), forest tree crops (Grattapaglia et al., 1995), and citrus (Ling et al., in press; Tozlu et al., 1999).

Tolerance to salt stress is a standard example of the quantitatively controlled trait in crop species. Breeding of crops that are tolerant to salinity is one of the primary goals for plant breeders. Soil salinization is a major process of land degradation, leading to falling crop yields and the loss of land from production in a range of environments

(Monforte et al., 1997). Human-induced salinization also contributes to degradation of world's dry lands and more temperate environments because it is closely associated with irrigation schemes. Citrus is a crop widely grown in the world and faces the challenge of soil salinization in many of the growing environments. Development of salt tolerant rootstock varieties of citrus is one strategy being pursued by citrus breeders. Like many other crops, citrus is able to produce a wide variety of genotypes through sexual hybridization, and many horticulturally important traits are inherited as QTLs, including superior growth in salty environments. In citrus, cold hardiness and salt tolerance are among the most significant QTL traits. Very importantly, these traits and their related physiological indexes are segregating in an intergeneric backcross population of *Citrus* and *Poncirus*. The previous QTL analysis in citrus has revealed 16 genomic regions involved in growth and dry mass production under both saline and non-saline conditions (Tozlu et al., in press), and 17 genomic regions are involved in Na^+ and Cl^- accumulation under saline and non-saline conditions (Tozlu et al 1999). The citrus genome map used in these studies was derived from a relatively small population of BC1 progeny with 60 individuals (Durham et al., 1992; Cai et al., 1994). In another intergeneric *Citrus* x *Poncirus* BC1 population, QTL analysis identified a major citrus nematode resistance gene region *Tyr1*, which explained 63% of the variance (Ling et al., submitted). All of the QTL analyses previously conducted were based on either a total genome map or a localized linkage map of citrus, comprised of RAPDs, RFLPs, and isozymes, and the marker density of the map was not as greater as the one currently constructed with AFLP markers, described in Chapter 3. Since the same population was used for both genomic maps, it is beneficial to integrate the two maps together, with more marker types and higher marker densities. Thus the advantage of higher marker density can serve to determine, reconfirm, and discover QTL regions for those growth- and salt tolerance-related traits.

The objectives of this study included: (1) To construct a high-density citrus genome map by integrating previously mapped molecular markers (Durham et al., 1992; Cai et al., 1994) and currently mapped AFLP markers; (2) To conduct a comparative study of map synteny; (3) To conduct QTL analysis for the genetic control of citrus growth-related and salt tolerance-related traits, under saline and non-saline conditions.

Materials and Methods

Plant Materials and Data Origin

The entire data set of RAPDs, RFLPs, and isozyme markers which was used for previous citrus genome mapping was obtained from the collaborative lab of Dr. G. A. Moore, University of Florida. The data were joined with AFLP marker data sets and rearranged by adopting only the markers reported on the core map of Cai et al (1994). Missing individuals were removed from each data set, so only those individuals with complete marker data were used for mapping. The joined data set consisted of 664 marker loci among 63 individual hybrids.

The QTL trait data were kindly provided by Dr. Moore. These data were generated using clonally propagated rooted shoots of parental plants and BC1 progenies, that were evaluated phenotypically for 38 traits under saline and non-saline conditions. A total of 22 salt tolerance-related traits and six growth-related traits were selected for the QTL analysis, using the integrated genetic map.

Marker Nomenclature

AFLP marker nomenclature included the letter E for the *Eco*RI primers and the letter M for the *Mse*I primers, each followed by selective nucleotides, and then the molecular weight of the polymorphic band analyzed. All RAPD markers were named OP followed by single or double letters and a number indicating their Operon kit origin, then the size of the fragment analyzed. The RAPD markers that came from the previous mapping study (Cai et al., 1994) were extended with the letter C. The RFLP data and isozyme data were designated as previously described (Durham et al., 1992). The RGC markers were all designated with RG, followed by their restriction enzyme origin, then with or without the fragment size. Two sets of data were generated jointly according to their marker genotypes. All of the aa x Aa genotype markers were used to construct a composite citrus genome map. An additional 89 AFLP markers, and 79 markers including RAPDs and RFLPs from previous mapping studies, were presumed to be of Aa x aa and Aa x Aa genotypes.

Linkage Analysis

Data were analyzed with JoinMap 2.0 (Stam and van Ooijen, 1996). A range of LOD threshold levels were tested to reach an optimum number of marker groups and marker ordering. Marker grouping processes were conducted under the minimum LOD score of 3.0 to 7.0. Finally, the LOD threshold 5.6 was chosen with a maximum recombination value of 0.49. Through the evaluations of the different grouping results, the markers that were mapped on the previous citrus linkage core map were primarily considered to be included in the new linkage groups. For each linkage group, recombination frequency was calculated, and pairwise data were generated for each of the markers in that linkage group. A suitable restrictive LOD threshold and recombination threshold values (10 times higher than suggested values) were used. The same sets of parameters were taken for the rest of the map construction procedures, as described in Chapter 3. The genetic distance was estimated using the Kosambi function.

QTL Analysis of Salt Tolerance Related Traits

The phenotypic evaluation results of twenty two salt tolerance-related traits and six growth-related traits, under both salinized and non-salinized conditions, were used for QTL analysis. The details of salinity treatments and statistical analysis of those phenotypic values was described in Tozlu et al. (in press). The abbreviations of the traits are presented in Table 4-1. QTL analysis was conducted using the currently constructed composite map and the data of 22 salt tolerance-related phenotypic measurements, and 6 growth-related measurements. The measurements were taken from different tissues of 48 available hybrids under salinized and non-salinized condition. Each plant was measured for their concentration of Na^+ and Cl^- in the different tissues. QTL analysis was carried out using MapQTL 3.0 (van Ooijen and Maliepaard 1996) which is compatible with JoinMap 2.0 (Stam and van Ooijen 1995).

Trait data were analyzed as a BC1 population. The entire genome was searched for putative QTLs with the interval mapping model. QTL detection for different salt tolerance-related traits was accomplished by scanning every two 2 cM of map distances throughout each linkage group. The search for putative QTLs of growth related traits were set for every 1cM throughout the entire genome. Three input files were prepared according to the computer program requirement. The "locus genotype file" for QTL analysis was adapted from the linkage analysis files of the combined map construction. The "map file" was obtained and modified from map construction results. The "quantitative data file" was prepared by modifying the format of the quantitative trait phenotype data. Although there were only 48 individuals for which quantitative trait data were available, the QTL analysis was conducted successfully. A minimum LOD threshold of 2.2 was used to declare the presence of a putative QTL in a given genomic region. The estimation of QTL effects was calculated where only one QTL at a time was allowed to explain the phenotypic variation in the trait, and the additive effect in both positive and negative senses were also calculated for each given locus genotype.

Results

Map Construction

A total of 552 (83.3%) of the 664 marker loci used for linkage analysis, were grouped under the LOD 5.6 threshold, and 21 primary linkage groups were established. After linkage group selection, 5 doublets composed of 2 AFLP markers each were eliminated from further map construction. There were 4 other small linkage groups (two of them with 4 markers, two with only 2 markers) remaining as minor linkage groups, because all of the markers in these groups were mapped on the previous citrus genetic map; they may be useful for the synteny analysis of the two linkage maps. Finally, a total of 509 markers (including AFLPs, RAPDs, RFLPs, RGCs, and isozymes) were used to construct a new citrus genome map with higher marker density. The map spanned a genetic distance of 1026.4 cM, distributed into 11 major linkage groups covering 965.4 cM, and 4 minor linkage groups covering 61 cM. The average genetic distance was narrowed to 2.0 cM between markers. The largest gap among the 11 major linkage groups was 15.6 cM, in linkage group VIII. The 11 major linkage groups of this combined genetic map have extended the genetic coverage by 106.8 cM, compared with the AFLP-based genetic linkage map (856.8 cM) constructed in Chapter 3.

Two of the minor groups (1 and 2) originated from the distal regions of linkage groups II and IV of the previous RAPD-based citrus linkage map (Cai et al., 1994), while the other two (3 and 4) came from the middle gap regions of previous linkage groups I and II, respectively. These minor linkage groups all originated from regions of substantial genetic gaps.

Merging of two maps by JoinMap 2.0 created a new linkage map with totally different linkage group orders (Figure 4-1A). Considering the original marker orders in the RAPD-based map, those previously dense regions were consequently more populated by AFLP markers (Figure 4-1B). The RFLP markers and isozyme markers in each linkage group of the previous linkage map were primarily considered as anchor markers to examine the new marker orders with newly introduced AFLPs in the combined map. The RFLPs and isozymes appear to be stable in terms of their orders in the composite map, thus they were used to monitor certain genomic regions (as marker brackets), as they were rearranged from the previous map into different linkage groups of the composite map. In almost all cases, as certain regions were being shifted into different linkage groups when AFLP markers were introduced, switched positions of the adjacent RAPD markers were observed. Although many previous marker clusters have been rearranged into different linkage groups on the new composite map by the insertion of additional AFLP markers, most of the marker brackets defined by RAPDs, RFLPs, and isozymes remained in the same clusters as in the previous map (Cai et al., 1994).

The AFLP markers greatly increased marker density of the linkage groups. The genetic gaps between those previously mapped markers were reduced significantly. The loss of some markers under different linkage analysis programs was observed when the two maps were compared. Markers OPE03160C, Pgd2, OPE16110C and OPG06098C, which were mapped at distal ends of previous linkage groups I, V, VI and VIII respectively, were lost in the joined mapping process with JoinMap 2.0. Markers pRLc60 and OPQ16046C were mapped in the middle region of linkage group III on the previous map, but they were also lost in the new mapping process. Furthermore, the markers Skdh and OPE05059C from previous linkage groups III and VII were not included in the data file, thus they were not shown in the new composite map.

Under the grouping module of JoinMap 2.0, some rather isolated marker regions on the previous linkage map were determined to be unlinked with any other linkage groups, and they formed new independent linkage groups. For example, markers OPT07072C and pgCit019 were mapped to the distal region of previous linkage group II, but they have formed a independent minor linkage group XII with two other AFLP markers. In the previous distal region of linkage group IV, markers OPG06023C, OPG11100C, OPE07070C, and OPF15063C were also separated as a whole bracket, and formed a minor linkage group for the new map. The original order of those markers was changed without additional AFLP markers in that linkage group. Two other new minor linkage groups were formed by the linked doublets OPE01054C/OPE04064C, and OPE20082C/pRLc39, which were mapped to the middle of previous linkage groups I and

II respectively; both of them were positioned in the middle of the gap regions. The regions of these two doublets were 13.6 cM and 24 cM away from their flanking marker regions of their linkage groups. The *Ctv* region, determined by previously identified closely linked markers was mapped to linkage group I; these markers including AFLPs, RAPDs, RGCs, and SCARs. The *Ctv*-linked marker array was unchanged in this composite map.

Map Synteny

Comparative study was conducted based on the two maps generated from different experiments and different mapping programs. The previous citrus genetic linkage map was based on RAPDs, RFLPs, and isozyme markers, and the linkage analysis program MAPMAKER/3.0 (Lander et al., 1987; Lincoln et al., 1992) was used to construct the linkage map (Cai et al., 1994). The current map was constructed based on the combination of AFLPs, RAPDs, SCARs, RGCs, RFLPs, and isozyme markers, and the linkage analysis was carried out by JoinMap 2.0 (Stam and van Ooijen 1995). Changes with regard to the generation of different linkage groups, and rearrangements in marker orders and genomic region locations, were observed. The comparison of the two maps was shown in Figure 4-1.

In the new composite map, linkage group I has covered a genetic distance of 98.9 cM, and included all of the markers in the region B of the previous linkage map. Introduction of the AFLP and other markers into this region has extended the genetic length of this region from 62.3 cM, and marker density was increased 3-fold for this region.

Linkage group II consisted mainly of AFLP markers, and this group has merged with most of the markers of previous linkage group VII, except marker OPE05059C, which was not included in the data set. The whole linkage group II has covered a genetic distance of 108.0 cM with 55 markers.

Linkage group III is the largest linkage group established in this mapping study. Linkage group III spans 199.1 cM, and contains 180 markers. This linkage group has merged several regions of the previous linkage map, including the O region (defined by markers OPF05105C, OPE19142C and OPG14059C), L region, E region (defined by pRLc31 and OPF04066), M region, D region and C region of the early map. Compared with the AFLP-based map constructed in Chapter 3, this linkage group has stretched from a total length of 148 cM to 199.1 cM. Two cDNA clones from the cold-acclimation-response, pBCORc115b and pBCORc115c, were mapped into this linkage group following the merger of the M region where they were originally mapped. The regional marker density for those merged genetic regions was increased in varying degrees (Table 4-2).

Linkage group IV is actually the expansion of the A region of the early map. After so many AFLP markers were inserted in that region, the original marker order was rearranged. The number of markers was increased from 17 to 44, and coverage increased from 74.5 cM to 99.1 cM.

Linkage group V corresponded to the Q region (linkage group VI in the previous map), except that marker OPE16110C was determined to be unlinked with rest of the markers. The Q region in the new map has been increased by 30 AFLP markers, and the length of the linkage group was expanded from 82.6 cM to 100.1 cM.

Linkage group VI corresponded to the G region in previous map. With 27 additional AFLP markers, the linkage group was extended from 57.7 cM to 68.8 cM. The co-segregating marker sets, pgCit042 and pgCit045, and OPA18064C and OPA12091C, remained as co-segregating sets.

Linkage group VII corresponded to region I of the previous map, but only 5 AFLP markers were added to this region. A new maker "saltt" mapped between marker OPA14032C and OPG18022. "Saltt" was a cDNA probe that was isolated from salt-tolerant citrus callus tissues. "Saltt" was not mapped on the previous map.

Linkage group VIII includes almost all the markers of previous linkage group VIII (R region), except marker OPG06098C. The R region was expanded from 18 cM to 42 cM, by the additional 8 AFLP markers mapped.

Linkage group IX is a new linkage group that only consisted of AFLP markers. In this linkage group 10 AFLP markers spanned 46.6 cM in length.

Linkage group X corresponded to the P region in the early linkage group V. New linkage group X included all the markers of P region with two additional AFLP markers, which were mapped distally to that region,10.7 cM apart from each other.

Linkage group XI corresponded with the H region of the early map. The isozyme locus Skdh, mapped in the middle of the gap region of the early map, was not mapped in the new linkage group XI due to missing data. One AFLP marker, EACMCTG230, was mapped on the distal position.

Minor linkage group XII was generated from the distal region of early linkage group II, defined by OPT07072C and pgCit019. The minor linkage group XIII was formed by markers OPG06023C, OPG11100C, OPE07070C, and OPF15063C, that were located in the distal region of the early linkage group IV. The minor linkage groups XIV and XV were produced from the two gap regions of early linkage groups I and II. These two minor groups were defined by markers pRLc39 and OPE20082C, and markers OPE01054C and OPE04064C, respectively.

QTL Analysis of Growth-related Traits

The QTL analysis was focused on six growth-related traits that were evaluated under both salinized and non-slainized conditions. QTL detection was achieved by the interval mapping model. A total of 36 putative QTLs, with various phenotypic effects, were identified for these traits; these were distributed mainly in linkage groups I, V, and VI. It is possible that some of the putative QTLs identified may be false due to the small size population used for QTL analysis. In agreement with a previous study, the criterion for determining the "true" QTLs was followed as described by Tozlu (in press), except the LOD threshold level was adjusted slightly lower due to the different QTL analysis program used. Briefly, when traits and their various derivatives had a LOD score > 2.2, and they constantly mapped to the same genomic location, it was considered more likely that the region contained one or more genuine QTLs.

For the trait Control Net Growth (CNG), there were four putative QTLs identified on the linkage group V and VI, with LOD score higher than 2.2. The QTL regions having the major phenotypic effects were located on linkage group V; the entire region had a range of LOD values from 2.2 to 4.4, and extended from map position 47.8 cM to 100.1 cM. The highest peak of LOD scores, 4.4, was presented in the interval region between marker EGTMCAT190 and ETAMCTT230, which explained 38.9% of the phenotypic variance of the CNG trait. Some other regions close to this major QTL interval were identified also, and they were indicated by the marker interval between ETGMCAT100 and EACMCTA370 (with LOD score of 3.9); and marker EAGMCAA185 (LOD 3.8) and marker ETTMCTG265 (LOD 3.72). Another QTL region, indicated by marker ETAMCAT410 at the distal region of linkage group V, had a highest LOD score of 2.9; it had a relatively smaller effect on phenotype variance for CNG, accounting for 24.9%. On linkage group VI, one QTL region for CNG was located between marker OPQ06089C and OPT06090C, with higher LOD scores of 2.4 toward marker OPQ06089C; this QTL explained 24.4% of the phenotypic variance.

There were six putative QTLs identified in association with the trait of Salinized Net Growth (SNG), distributed in linkage groups I, V, and VI. The QTL with the greatest phenotypic effect on salinized net growth was located in linkage group VI, indicated by

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marker OPQ06089C with the highest LOD score of 5.4 for this locus, and explained 42.5% of the phenotypic variation. On linkage group VI, there is a lesser QTL region (25.4% phenotypic variance explained) detected with LOD score of 2.4, located between marker OPT06090C and EACMCAC200. Three QTLs with minor phenotypic effects for this trait were located on linkage group V, their LOD score range from 2.2 to 2.7. The three QTL intervals and the highest LOD score peaks were indicated by markers ETGMCAT190, EACMCTA370, and ETAMCTA520, respectively.

The responsive index for the net growth trait under the salinized condition of the citrus hybrids was measured as the ratio of SNG/CNG. The phenotypic analysis showed that growth of all hybrids decreased due to salinity treatment (Tozlu et al., in press). In this new composite map, there were four genomic regions identified in association with this trait (index), and they were located in linkage groups I and VI. Three regions in the linkage group I have LOD scores higher than 2.2. The first one was detected near OPQ17064C with a LOD score of 2.3, and this QTL accounted for 23.9% of the phenotypic variation. The second one was found in the interval between marker EACAMCTT260 and EACAMCTT130 with LOD peak of 3.6 towards to marker EACAMCTT130, accounting for 30.6% of the phenotypic variance. The third interval in linkage group I with the highest LOD score of 3.9 was flanked by marker ETGMCAG220 and EACAMCTT140, and explained 36.4% of the phenotypic variance. Another interval for SNG/CNG was detected on linkage group VI, located on marker OPQ06089C with peak LOD of 2.5, and explaining 22.4% of the phenotypic variance.

The genetic additive effects of these QTL regions located on the linkage group I were all negative, which means the phenotypic value decreased when these loci were in the heterozygous state (presence of the *Poncirus* alleles, for salt sensitivity). On the contrary, the additive effect for the QTL regions of SNG/CNG trait on linkage group VI was positive, indicating an allele for salt tolerance.

The total growth of both salinity and non-salinity conditions were taken as the experiment terminated (Tozlu et al., in press). There were six putative QTLs for the trait Control Total Growth (CTG) found on linkage groups V and VI. The QTL with highest LOD scores of 3.8-3.9 was identified at the interval of markers EACMCTA370 and EAGMCAC170. The genetic distance of this interval was 1.1cM, and 34.3% of the phenotypic variance was accounted for by this region. Two QTLs were located in two intervals compartmentalized by markers ETGMCAT190, ETAMCTT230, and EACTMCTC280, with LOD scores of 3.5 and 3.2 respectively; these two QTL regions were 1.7 cM apart on the map. Markers ETTMCTG265 and ETAMCAT410 were in the position where two putative QTLs were located, both with LOD scores of 3.1. On linkage group VI, the region between OPQ06089C and OPT06090C also contained one QTL with smaller effect on CTG, accounting for 27.1% of the phenotypic variance, with an LOD score of 2.6 at the peak.

Under the salinity condition, six putative QTLs were found related to the Salinized Total Growth (STG) trait, distributed in linkage groups V, VI and VIII. On linkage group V, one putative QTL was detected around the marker EACMCTA370, with an LOD score of 2.6. Linkage group VI had a QTL with large phenotypic effect (36.4%), and another QTL with relatively smaller phenotypic effects (30%); the former had a higher LOD score of 3.96, while the latter one had a LOD score of 2.9. The major QTL region was located between markers OPQ06089C and OPT06090C. The QTL with smaller effect was located in the region between markers OPT06090C and EACMCAC200. The two QTL regions (two LOD peaks) were approximately 7.5 cM apart. Three other putative QTLs were detected on linkage group VIII. The RFLP marker pRLc91 has identified a minor phenotypic effect QTL, with a LOD score of 2.2; two other smaller effect QTLs were positioned by markers ECAMCTC150 and ETAMCTG340, approximately 10.2 cM from each other. There was no indication of a salt-tolerance related QTL present in or around the marker "saltt", which was derived from a cDNA probe isolated from salt tolerant callus tissue of citrus.

The total growth trait responsive index for the salinized condition (STG/CTG) was also tested for responsive QTLs on this map. There were four putative QTLs for this trait detected in linkage group I, with rather lower LOD scores compared with other traits. The first one was found in the interval between markers OPQ17064C and EACAMCTT260, with a LOD score of 2.2. The second QTL for this trait was located in a rather narrow region defined by markers EACAMCTT260 and EACAMCTT130, with highest LOD score of 2.4; these two markers were 1.8 cM apart. The third QTL was present in the 4 cM interval between markers pRLc32 and ETGMCAG220, with a LOD score of 2.3. The fourth QTL region was represented by marker EAAMCTT290, with

LOD score of 2.2. The overall low LOD scores and low phenotypic effects on this trait in some ways may imply that STG/CTG does not respond very significantly to salinity like other traits; on the other hand, the measurement of total growth may not reflect the genetic response to salinity as well as net growth measurement does.

On linkage group I, the genomic region defined by markers EACAMCTT260 and EACAMCTT130 harbored a major QTL for STG/CTG, and the same region also possessed a putative QTL for SNG/CNG with relatively high LOD scores. The genomic region in linkage group V, designated by marker EACMCTA370, had a putative QTL for CNG. In the same region (represented by EACMCTA370) the putative QTL with highest LOD peak in linkage group V was found for CTG, SNG, and STG, and this region was the major QTL region for CTG. In the same linkage group two other regions designated by two markers, ETTMCTG265 and ETAMCAT410 respectively, also harbored putative QTLs for CNG and CTG. The region in linkage group VI around marker OPQ06089C has putative QTLs for CNG, CTG, and SNG/CNG. The putative QTLs for these 6 growth-related traits were detected mainly on linkage groups I, II, III, IV, V and VIII. The locations of the putative QTLs for each trait, the LOD scores, percentage of phenotypic variance explanation, and additive effects were all summarized in Table 4-4. The genomic locations of the putative QTLs for those growth-related traits were indicated in their corresponding linkage groups in Figure 4-2.

Salt Tolerance-related QTL Analysis

QTL analysis was conducted using all the markers on this newly constructed composite map. The LOD threshold of 2.2 was used to declare the presence of putative QTLs. There were 15 of 22 traits found to have responsive putative QTLs on various linkage groups. Totally, 50 responsive putative QTLs were identified for these 15 salt tolerance-related traits.

For many traits tested, in a single linkage group there was more than one LOD peak (LOD > 2.2), presenting linked putative QTLs in a continuous fashion. In such cases, each LOD peak was marked and the percentage of the phenotypic variance contribution was examined. Major QTLs were declared according to the highest LOD peaks and the phenotypic variance contribution from that region. When two linked LOD peaks were observed with an overlapping LOD >2.2 support interval, only one QTL was declared in the region at the location of the highest LOD peak. The consistency of the LOD scores flanking a marker or region with the LOD peak was carefully evaluated along the map position, and taken into account for the determination of the presence of a QTL.

The putative QTLs for these 15 salt tolerance-related traits were detected mainly on linkage groups I, II, III, IV, VI, IX and XIII. The locations of the putative QTLs for each trait, the LOD scores, the percentage of phenotypic variance associations, and additive effects were all summarized in Table 4-3. The genomic location of the putative QTLs were summarized in Figure 4-2.

The genomic regions that harbored putative QTLs for more than one trait were frequently observed. In the linkage group III, QTLs with various phenotypic effects for traits STNa, SlfNa, STCl, and SlfCl were identified in the same genomic area indicated by marker pRLc32; the QTLs for traits STCl and CTCl were both found in the genomic region of marker OPQ17064C. On the linkage group III, there were more overlapping QTLs for different traits in the same genomic regions. The QTLs for CLfCl and CSmCl, with different levels of phenotypic effects, were all defined by the same markers. The highest LOD peak of 7.7, for the trait CLfCl, was indicated by markers ETTMCTA195 and EACMCAA185; the second LOD peak of 5.8 was indicated by marker EAGMCTA185; and the third LOD peak of 5.2 was indicated by EAAMCTT205. For CSmCl, the same LOD peaks were found at the same positions on the linkage map as for CLfCl, with slightly different LOD values (5.9, 4.1 and 3.7 respectively). For these two traits, even their three “ghost QTLs” (the putative QTLs without the LOD scores consistency around the region) were identified at the same locations. There was a minor QTL for CTCl with an LOD score of 2.4, located by marker EAAMCTT205; and a minor QTL for SLfCl was also found at the same position, with an LOD score of 2.6.

On linkage group IV, three QTLs (including QTLs with major effects) were identified which were responsive to the traits STCl_Na and SlfNa. There were QTLs with minor effects for trait SsrNa which were located also in the same genomic region. These responsive QTLs were all located in the genomic region designated by a block of RFLP markers (pgCit037, pRLc66L, pgCit039, and pcPt001) and markers ETGMCTG240 and

EACAMCAG80. The genomic region defined by markers OPE19066C and pgCit057L harbored a QTL with major effects for the trait SsrNa, and one of the minor effect QTLs for trait SLfNa. There were two QTL regions identified for trait SSmCl in linkage groups IX and XIII. The linkage group XIII carries the major responsive QTL for SSmCl with an LOD score of 3.3. In the linkage group I, the RGC marker RGpt8taqI has indicated one putative QTL for trait SSrCl, with an LOD peak of 2.7.

There were 8 indicated regions (LOD > 2.2) that were questionable for the true QTL existence, because of the inconsistency of the LOD scores detected in flanking regions. Such regions either demand more markers for QTL analysis or might be considered as "ghost" QTL regions. For instance, for the trait CLfCl, there were three regions on the linkage group III with LOD peaks flanked by steeply reduced LOD scores (less than 0.1); such regions were not considered as significant QTL regions for the trait. The same was detected for CSmCl at almost the same region of the linkage group III. Finally, there were seven salt tolerance-related traits for which no responsive QTLs were found on this composite map by MapQTL 3.0. They were all listed in Table 4-3.

Discussion

Map Integration and Map Synteny

By integrating the genetic maps from two different mapping studies, a new citrus map was generated, composed of different types of molecular markers, and produced by two different mapping programs. The map saturation powers of AFLP markers were demonstrated again in this map integration. However, it was also revealed that more markers are needed for ideal citrus genome map construction. Further, the disadvantage of the small size population was demonstrated by the large scale rearrangement of the linkage groups, and the fact that the number of linkage groups obtained was more than the citrus haploid chromosome number. Although saturation of the linkage map was not achieved, certain genomic regions were more densely populated by AFLP markers. The differences of map length between these two mapping studies may reflect the different criteria that are being used internally by the two different mapping programs for the linkage determinations. Another cause of genetic distance differences could be the additional markers, because adding markers causes reordering positions on the map, and different genetic distances for suitable new marker recombinant frequencies result.

By joining two data sets together, the total map length of the resulting composite map has stretched 106.8 cM longer in the major linkage groups, compared with the AFLP-based map (in Chapter 3). In similar manner, increased map lengths were reported in combined mapping of barley (Becker et al., 1995), rice (Maheswaran et al., 1997), sugar beet (Schondelmaier et al., 1996), and sorghum (Boivin et al., 1999). On the basis of the few studies reported to date, the stretching of the map seems to be independent from the distribution of AFLP markers along the genome, which implies that stretching of the map may also be independent from the restriction enzyme applied. The evidence for this suggestion came from the barley mapping studies. The barley genome has a nearly random distribution of AFLP markers, but map length was increased from 1096 cM to 1873 cM (Becker et al., 1995). In a rice mapping study of *Pst*I-based AFLPs, stretching of the map from 1811cM to 3058 cM was observed (Maheswaran et al., 1997). By contrast, although the AFLP clusters were found on the sugar beet map, no stretching resulted (Schondelmaier et al., 1996). The results from the citrus maps have suggested that stretching of the map may be due to unsaturation of the linkage groups, because most of the previously marker-populated regions were populated further by AFLPs, and the previous genetic gaps were less likely to be more densely populated. This finding also indicates uneven distribution of AFLP markers by the *EocR*I/*Mse*I restriction enzyme system. Compared with the map generated by Cai et al. (1994), a shorter citrus map with higher marker densities was obtained.

In another parallel comparison, this composite map provides less genome coverage than the RAPD-based map (Cai et al., 1994), probably because of the different calculations for linkage establishment by MapMaker and JoinMap. The previous mapping study used fewer markers but resulted in 70-80% the citrus genome coverage with few genetic gaps (Cai et al., 1994). Exploration of AFLP markers lead to the detection of new recombination events, but use of the same size population may be incompatible with the higher marker number situation, and might have resulted in non-significant linkages for some markers. Such situations have trigged further rearrangement of previous genomic regions and marker orders, the loss of a few regionally isolated markers, and the formation of minor linkage groups because of lacking bridging recombinant markers to merge them with major groups.

It was observed that RFLP and isozyme markers in this composite map have well maintained their orders as in the genetic map of Cai et al (1994). To avoid the marker disorder caused by unknown linkage phases (coupling and repulsion phases) of the dominant markers (AFLPs or RAPDs), inclusion at least one or two RFLP markers (co-dominant markers) in each linkage group was suggested (Schondelmaier et al., 1996). In our mapping study, intermingling of the AFLPs, RFLPs, and RAPDs were observed in linkage groups I, II, III, IV and VIII. Linkage groups V, VI, VII, X and XI had less intermingling of AFLPs with previously mapped markers, and most of the AFLP markers were added to the distal regions of those linkage groups.

In the comparison of genomic regions of these two maps, many markers of the previous map have switched their positions into different genomic regions of the composite map due to the possible reason stated earlier. However, the cohesion of those markers in their relative position was retained, regardless of the large number of newly-joined AFLP markers inserted between them or extended in their flanking regions. In general, the regions that were populated with previously mapped markers were likely populated further with newly added AFLP markers. The integrity of most genomic regions was well conserved, as was proven by subsequent QTL analysis. The markers that were rather isolated from a populated region in the early map were either lost in the new map or formed new linkage groups with the addition of AFLP markers. There were five genomic regions of the previous map merged into linkage group III of the composite map. This implies that the additional AFLP markers have included some that functioned as a bridge for those regions, and facilitated the merger of those markers from previously distinct linkage groups.

Although the total genomic coverage of the composite map does not reach the same coverage as the previous RAPD-based map (Cai et al., 1994), AFLP markers have significantly filled those gaps within the marker populated region, and greatly extended distal regions of a few linkage groups. Considered regionally, the addition of AFLP markers increased genomic coverage for all the corresponding genomic regions of the earlier map of Cai et al. (1994). Several large gaps still remain to be filled, and a few markers isolated from previously populated regions were lost, due to lack of linkage with other markers. This result implies that strong localized crossing-over regions do exist within the F1 hybrid genome of the donor parent for this intergeneric backcross population. If the linkage gaps of the previous map were due to the lack of polymorphic RAPD markers in some genomic areas having a higher frequency of crossing-over, then the capability of adding AFLP markers to such areas can be considered a positive attribute of the AFLP marker system, even though the putative localized high frequency of crossing-over events still produce large centiMorgan estimates. The evidence of strong localization of crossing-over in specific cross combinations was noted and discussed by Becker et al. (1995), Nilsson et al. (1993), and Säll and Nilsson (1994). Nilsson et al.

(1993) pointed out that gaps caused by the strong physical localization of crossing-over might be an indication of the inconsistency between chiasma counts and map length.

Combining AFLPs and other types of markers produced a relatively more evenly distributed linkage map than the non-combined maps. However, in most of the linkage groups, clustering of large numbers of the AFLP markers was observed. Contrary to the observations in other studies (Becker et al., 1995; Schondelmaier et al., 1996; Boivin et al., 1999), there was no tendency of AFLP markers to flank the RFLP clusters.

In summary, the addition of AFLP marker loci to the original RFLP map has increased the marker density of the map and generated a good correlation between the number of markers per linkage group and the centiMorgan length of the linkage groups. The composite map now has increased regional genetic coverage due to the bridging of the gaps within corresponding regions, and in addition to distal regions of some linkage groups. Increasing the regional density by AFLP markers should be beneficial to the following QTL analysis by allowing more accurate localization of the QTLs on the linkage groups. In order to have the linkage group number reach the citrus haploid chromosome number with nearly the full coverage of the citrus genome, more co-dominant type markers (RFLPs, SSRs, or SCARs) should be applied to this study, and if possible a larger population should be used. Although in our study, AFLP markers were intermingled with RFLPs and RAPDs, it cannot be concluded that AFLPs, RFLPs, RAPDs, and other type of markers have indeed marked different regions of the citrus genome.

QTL Analysis of Traits Related to Growth and Salt Tolerance

A new level of marker density was applied to this QTL analysis. The QTL analysis result for the six growth-related traits has indicated the existence of genuine QTLs. The high-density map has resulted in more precise positioning of QTLs in a given region, and it is advantageous for the analysis of overlapping QTLs for more than one trait.

It was very obvious that almost every putative QTL region detected in this study was able to be narrowed down to individual markers, which define the interval closely. An interesting region was presented in linkage group VI with LOD score of 3.1, which is greater than the threshold value. It was defined by marker ETTMCTC150, but the LOD score of its flanking regions were very steeply reduced to zero, which showed this region to be very isolated. Such incidents may imply lack of markers in or around that region for QTL, or the presence of a "ghost" QTL. As the results summarized the QTLs for growth related traits, the overlapping of some putative QTLs in a particular region was observed in linkage group I, V and VI. The overlapping of QTLs is very critical for determining the genuine QTLs, and it is also important for the classification of various types of QTLs. According to the QTL study for salt tolerance in *Lycopersicon* species by Monforte et al. (1997), QTLs were classified into four categories: "Constitutive QTLs", those that showed no important change in their gene action and were detected in both non-salinity and salinity conditions; "Altered QTLs", those that were detected under both conditions but where the degree of dominance changes depending on the presence/absence of salinity; "Response-sensitive QTLs", those only detected under non-salinity condition or those whose contribution to the phenotypic value significantly decreased under conditions of salinity; and "Response-tolerant QTLs", those only detected under salinity or those where there is a change in the direction of gene effects depending up on the presence/absence of the salinity.

In this QTL analysis, the searching for putative QTLs was conducted by the interval mapping method, and genomic regions were tested for QTL existence by scanning every 1cM of the entire genome. The advantage of a higher density genome map has certainly enhanced such QTL search. In general, the positioning for most of the putative QTLs in the map can be easily referred to those markers with highest LOD peaks or the marker close to it on the map.

The summary of the putative QTLs for these six growth related traits was presented in Table 4-4 and Figure 4-2 which indicated their relative position on this genomic map. It is difficult to compare our QTL analysis results with the classification criterion for QTLs types as described by Monforte et al (1997). Because whether the significance differences of the phenotypic value explanations increase or decrease by different traits was hard to determining, and the partitioning of total variance into genetic variance was not done, the real QTL function cannot be determined precisely. Thus the comparisons with good confidence were only those comparisons between major QTLs for one trait and minor QTLs for others. In growth-related trait QTL analysis, the overlapping analysis results of only the major QTLs for trait CNG (in linkage group V) seem to meet the criterion of "Response-tolerant" QTL.

There were eight genuine QTLs identified for growth-related traits CNG, SNG, STG, CTG, SNG/CNG, and STG/CTG. Analysis of overlapping QTLs revealed close linkage between some QTLs for different traits. For example, in linkage group I, a genomic region defined by markers EACAMCTT260 and EACAMCTT130 harbored a major QTL for trait STG/CTG. In the same region a putative QTL for trait SNG/CNG with relatively high LOD scores was also detected, their highest peak points being 0.8 cM away from each other in the map. Another incident was found in linkage group V with the genomic region designated by marker EACMCTA370 representing a major putative QTL for trait CNG with highest LOD score 3.9. In the same region (represented by EACMCTA370), the putative QTL with highest LOD peak 3.9 for trait CTG was also found, except the LOD peak position was shifted 1.3 cM away from the LOD peak of CNG trait. Such presentation may indicate that these two QTLs were closely linked. The same region was found to have relatively minor effect QTLs for traits SNG and STG, also indicating close linkage of these QTLs. Possibly, the salinized condition has turned on another set of genes that function as minor QTLs for trait SNG and STG. The same conclusion could be extended to two other regions designated by two markers ETTMCTG265 and ETAMCAT410, respectively, where putative overlapping QTLs for traits CNG and CTG were found. The detection of QTLs in the region of linkage group VI around marker OPQ06089C for trait CNG, CTG, and SNG/CNG also is in agreement with the above conclusion. The traits CNG and CTG shared most of same QTL regions. It is not possible, at this point, to distinguish effects of pleiotropy and tight linkage of QTLs. To distinguish the pleiotropy effects and the effects of tightly linked QTLs, an analysis would require a larger population and phenotypic evaluation of more progeny, greater numbers of linked markers, and most of all, the availability of qualitative gene markers that are linked with quantitative loci.

For the salt tolerance-related traits, genuine QTLs were also determined by the same criteria used for growth related QTLs. The genuine QTLs were summarized in Table 4-3. The following results further proved the existence of genuine QTLs. QTL analysis has shown that the marker region of pRLc32 hosted putative QTLs for STNa, SlfNa, STCl, and SlfCl. The genomic region designated by marker OPQ17064C also carried QTLs for STCl and CTCl. The overlapping of QTLs may suggest the same gene(s) are responsible for the Na^+ and Cl^- content changes in the whole plant and in leaf tissue, under the salinized condition. The same suggestion could be extended to CLfCl and CSmCl.

The traits CTCl_Na and STCl_Na measure the ratio of Cl^- and Na^+ in the whole plant under the unsalinized and salinized condition, and it seems that different genes are responsible for these two traits. There are more responsive QTL regions for STCl_Na than CTCl_Na, and the level of the LOD significance for STCl_Na was greater than that of CTCl_Na. This result implies that the gene expression level for Cl^-/Na^+ in the whole plant differed significantly under salinized and non-salinized conditions. According to the

QTL classification criterion (Monforte et al., 1997), the genuine QTL for these two traits seems to fall into the category of "Altered QTLs". Very comparably, in linkage group IV, three QTLs (including major QTLs) responsible for STCl_Na, the three correspondent QTLs for trait SLfNa, and the minor QTLs for trait SsrNa were all located in the same genomic region. The major QTL for the trait SsrNa and one of the smaller effect QTLs for trait SLfNa were defined by markers OPE19066C and pgCit057L. These findings suggest that the same sets of genes are responsible for these three traits under salinized condition, with different levels of reaction to Na^+ regarding leaf tissues and structure roots.

There were 8 indicated regions ($LOD > 2.2$) that were questionable for genuine QTL existence, because the inconsistency of the LOD scores detected flanking the regions. These regions either demand more markers for QTL analysis or might be considered as "ghost" QTL regions. Such regions were detected for CLfCl and CSmCl in three regions of the linkage group III, their LOD peaks were flanked by steeply decreased LOD scores (less than 0.1). The cause of the regional LOD score inconsistencies cannot be determined firmly at this point, but one possible reason is the lack of significant numbers of markers in and around those regions.

Markers Associations with Phenotypic Variance

In this study, the percentage of the phenotypic variance explained by a given genomic region (LOD peak region) was taken into account to distinguish QTLs with major or minor effects. The phenotypic variance detected by a single putative QTL ranged from 20.2% to 52.3% for the traits tested in this study; these percentage values seem reasonable for this population size. Normally with a larger population, the amount of the phenotypic variance explained by a single detectable QTL will drop significantly. In a maize QTL study, it was reported that some markers accounted for only 0.3% of the phenotypic variance with the population size of nearly 2,000 individuals (Edwards et al., 1987); and with a population of 300 individuals, 4% of the phenotypic variance was accounted by QTLs (Doebley et al., 1991). Percentages of 7.1% to 53.1% of phenotypic variance accounted for by markers were reported in the QTL study of morphological traits in maize, with a population of 150 individuals (Veldboom et al., 1994). In tomato QTL study, 12% to 59 % of the phenotypic variance explanation was reported by Grandillo and Tanksley (1996).

Salinity Effects and QTL Stability

The choice of optimum selection environments has been controversial for salt tolerance. Testing subsets of the environments, including stress and non-stress environments, for the selection of the stress tolerance has been suggested for breeding of "wide adaptation" for the increase of crop yields (Igartua 1995). In the tomato study, Monforte et al (1997) pointed out that the optimum selection environment must be focused on whether, in spite of differential gene expression, selection under non-saline conditions can ensure a minimum response under saline conditions; or conversely, genes acting in the non-saline condition are different from those acting under salinity, thus causing the selection under non-salinity to be inefficient. QTL analysis with a high-density map may be an alternative approach to study the salt-induced differential genotype expression, or genotype x environment (G x E) interaction. The traits and QTLs studied were based on phenotypic evaluations conducted under two different environments (Tozlu et al in press), and the G x E interactions could be revealed with a compatible analysis model. With the enhancement of high-density markers on the map, QTL analysis helped elucidate the complexities of the citrus genome to some extent. Previous studies have shown that a unique array of QTLs may be detected in different environments and populations, with some QTLs being stable across environments (Paterson et al., 1991; Schon et al., 1993) and populations (Beavis et al., 1991). A consistency of maize QTLs across environments was demonstrated by Stuber et al. (1992), who suggested that major QTLs could be reliably detected in different environments. In this citrus study only one population was used, but many QTLs detected appeared to be stable across two environments (unsalinized and salinzied), and others detected were founded to be inducible by salinity. Further research using more than one population, and additional individuals in the populations, should be beneficial for QTL mapping of other traits in citrus.

Relating Quantitative and Qualitative Inheritance

In citrus there are many QTLs and single functional genes that remain to be uncovered and mapped on to linkage maps. As more genes of both types are identified, it will become possible to explore the relationship of quantitative and qualitative inheritance. The establishment of connections between quantitative and qualitative inheritance will have great impacts on genomic manipulations all crops, including citrus. So far, no QTLs have been found which coincide with regions or loci defined by alleles with qualitative effects. However, there are only a few qualitative trait loci identified and mapped in different populations in citrus, and only a very small portion of the quantitative traits have been analyzed. The linking relationship between quantitative and qualitative genetic variation was suggest by Roberston (1985), which briefly stated that alleles with either quantitative or qualitative effects should be expected to reside at the same locus. The primary data supporting this hypothesis were reported in *Drosophila* (Mackay and Langley 1990), and supportive observations of association between loci defined by plant mutants and QTLs were obtained in maize QTL mapping studies (Beavis et al., 1991; Edwards et al., 1992; Veldboom et al., 1994). Citrus, so far, has insufficient established data available to reveal any linking relationship between quantitative and qualitative traits. A single, RAPD marker-defined locus representing a major QTL region for citrus nematode resistance was reported in QTL analysis of another intergeneric backcross population (Ling et al., in press). The QTL analytical approach suggests an important advantage to gene mapping, and conjunction between quantitative and qualitative genetics by identification (or searching) of links between QTL regions and the loci defined by alleles with qualitative effects. Through the means of molecular markers, many loci can also be defined by recovery of alleles without evaluation of qualitative effects, and many new loci also can be defined by large scale mapping of cDNA clones or BAC clones. It is possible that BAC clones (or BAC end sequence-derived markers) which are directly associated with QTL effects being mapped with other markers, can serve for marker-assisted selection. Large scale sequencing, however, is necessary for assigning biological significance to such clones, especially for those genetic locations that lack loci defined by phenotypic values. QTL mapping may also provide genetic insight into traits and biological processes that may not be readily defined by known responsive cDNA clones. In this citrus study, one cDNA clone "saltt", obtained from salt tolerant citrus callus, was mapped, but none of the salt tolerance-related QTLs were found in or around this locus. QTL mapping may, therefore, represent crude but efficient genome-wide assessment of regions and their functional significance for various traits. QTL mapping also may lead to bridging quantitative and qualitative genetic complexity.

QTL Mapping and Marker-assisted Selection

The backcross population-based QTL mapping strategy involves the construction of genetic linkage maps of molecular markers and the identification of the QTLs for individual phenotypes. This approach is particularly attractive because it can reduce the negative effects of the linkage equilibrium often encountered in the marker-assisted breeding of outbred species (Grattapaglia et al., 1995) such as citrus. The association between markers and traits established in one cross would not hold in the second pedigree, because the markers and QTL alleles would be randomly associated at the population level (Soller, 1978; Beckman and Soller, 1983; Lande and Thompson, 1990; Strauss et al., 1992). In the present study, the parents used in the backcross have involved two pedigrees. The alleles of interest and their linked markers appear to be stable, and the results have shown that within-family linkage disequilibrium can be used to identify genomic regions controlling quantitatively inherited traits related to growth and salt tolerance in citrus. This result is important, because this approach can be used to perform QTL analysis in many available backcross families generated from highly heterozygous or distantly related species (intergeneric), like *Citrus* and *Poncirus*. For tree crops, this approach opens the possibilities of using already existing populations in the field for QTL analysis of many other quantitative traits. With the availability of powerful and easy to use QTL analysis programs, such as JoinMap 2.0 and MapQTL 3.0, the full-sib open pollinated populations also can be analyzed with the CP type (open pollinated population type) analysis model (van Ooijen and Maliepaard 1996).

In spite of the heterogeneous nature of the QTL mapping population and the limited sample size, we were able to map a number of QTLs controlling a significant proportion of the phenotypic and genetic variation in traits related to growth and salt tolerance. The discovery of overlapping putative QTLs demonstrated that traits under investigation in these studies were under the control of few major genomic regions with large effects, and accounted for most of the variation observed. The results of the QTL analysis suggested that the salinized condition can play an important role for differential gene expressions. With the higher density genetic map available, QTL identification of targeted traits should provide more powerful markers for marker-assisted selection. Molecular marker-assisted breeding for plant growth and salt tolerant traits would be a highly desirable way to track the inheritance and segregation of important genomic regions on an individual basis. The experiment conducted under salinity and non-salinity (Tozlu 1997), the detection of different QTLs (or differentially expressed QTLs) upon the presence/absence of salinity could be used in the design of a marker-assisted selection scheme which, taking into account all of QTLs of the trait, would help to broaden "adaptation" from non-saline to saline condition in citrus. Such understanding should substantially accelerate the introgression of the beneficial QTLs into breeding populations, as well as to facilitate the indirect pre-screening of individual hybrids, thereby reducing the cost and time of the conventional hybrid assessment for superior growth and salt tolerance.

For the selection of salt-tolerance citrus rootstock, one should consider the selection environments (salinity and non-salinity environments) as pointed out in the tomato salt-tolerance genetic study (Monforte et al., 1997). In our study, these two environments were used for phenotypic evaluation of all six growth-related traits. For the marker-assisted selection of salt-tolerant citrus rootstocks, the markers that facilitated growth-related QTLs under both salinity and non-salinity conditions should be considered, because QTLs of growth-related traits represent the more general recognition of the plant growth under the influence of those QTLs under both environments. On the other hand, the relationship between each of the QTLs for salt-tolerance-related traits expressed in different plant tissues and the general salt tolerance responses remain to be studied further. The QTL results of our study in those growth-related traits are rather more generalized and directly relate to salt-tolerance responses, and are more easy to be referenced for marker-assisted selections. Based on our results, the major QTLs for traits CNG and SNG were located in the same regions of the linkage group V and VI (except linkage group I which had QTL peaks present for SNG but with negative additive effects). The linkage group VI has higher LOD scores (5.42) than other regions, and has a higher percentage of phenotypic variance explained (42.3%) by this region for trait SNG. This region was indicated by marker OPQ06089C. On linkage group V, the regions in and around the markers ETGMCAT190, EACMCTA370, ETAMCTA520, ETTMCTG265 showed higher LOD peaks and higher percentages of the phenotypic variance explanation values than other markers in the region for trait CNG, CTG, SNG, and STG. On the other hand, the growth-related QTLs were identified on linkage group I at the same genomic region in or around the marker OPQ17064C, pRLc32, ETGMCAG220, and EACAMCTT140 for traits SNG, STG/CTG, and SNG/CNG. However, all of them presented negative additive effects for those traits. This result means the presence of the *Poncirus* alleles decreased net growth under the salinity condition. The finding of these above mentioned molecular marker regions can provide useful information for citrus breeders to facilitate the marker-assisted selection for salt-tolerance citrus rootstock, according to both saline and non-saline conditions.

In order to pinpoint the QTL position, defining the confidence interval should require a much larger population for QTL mapping. Darvasi et al. (1993) concluded that the confidence interval for QTL map location can be rather broad, in some cases essentially covering the whole chromosome, and may be relatively independent of marker density. Although our map has higher marker density, since the population was rather small, the accuracy of the QTL locations still is not beyond the level of assigning them to linkage groups. The QTL positions reported in this study were therefore tentative. While the issue of precise QTL position would be crucial for map-based cloning efforts, the current map should be useful for the further marker-assisted selection for favorable QTL alleles. Finally, given the time required to generated AFLP markers, the ones we have adapted here show great potential for accelerating the search for QTLs (in adequate populations) and could have good applications in marker-assisted selection using advanced back-cross schemes (Tanksley and Nelson 1996).

Conclusions

In this integrated mapping study, we have successfully integrated two sets of several types of markers into a total citrus genome map. The saturation power generated by AFLP was demonstrated in this study. Although the total genetic coverage of the composite citrus map seems shorter than the one previously generated (Cai et al., 1994), all of the marker populated regions were populated more densely by AFLP markers. Creating a valid high-density genomic region by introducing AFLP markers was proved feasible, as shown by the stability of the *Ctv*-linked marker region that included AFLPs and other types of markers identified in previous experiments.

The stability of some important markers was examined by introducing different types of markers into the same genomic region. Most of the RFLP markers and bulk segregant-identified marker loci were stable and remained in the same position, even though their entire linkage group was shifted or merged into new locations. The merger of previous linkage groups into a single larger linkage group, as well as breakage of some previous linkage groups, demonstrated the need for more markers and for increasing the population size for saturated citrus genome map construction.

Map synteny analysis reflected differences arising from the calculations for linkage determination by MapMaker and JoinMap mapping programs; specifically JoinMap generated shorter linkage groups than MapMaker. The integration of two available maps together has created a high-density map base for QTL analysis. The advantage of using a high-density map for QTL analysis in citrus was demonstrated by more accurate locations of QTLs by markers. The QTL analysis of salt tolerance- and growth-related traits provided insight to the citrus genome complexity for salt tolerance. The possible genetic linkage relationship between some of the QTLs was pointed out, and the possible interactions between QTLs were reflected by the results of the QTL peak values and PVE (Phenotypic Variance Explanation) values, as well as the additive effects in both positive and negative senses. These results are the first to relate AFLP markers with QTL traits in citrus, in conjunction with other types of markers.

The JoinMap compatible QTL analysis program MapQTL 3.0 was easy to use and suitable for conducting QTL analysis of the integrated maps, and it is able to analyze many other population types. These functional characters are important for citrus, because integration of citrus linkage maps from different mapping experiments and combination of available molecular marker types is possible and useful in citrus genetic studies. The integrated citrus genome maps would have extended genetic information regarding more traits (including QTLs) if those traits can be evaluated phenotypically, and QTL traits can be located onto the integrated citrus maps by QTL analysis program, such as MapQTL 3.0. Furthermore, there are many open pollinated or inter-related populations available in the field, and many of their quantitative traits can be measured for QTL analysis. MapQTL 3.0. is able to analyze CP type (open pollinated type) populations, and such capability will enable identifying more conserved or stable loci of horticultural interest, across the different populations in citrus. It would be beneficial if qualitative loci can be identified in linkage with identified QTL regions.

Table 4-1: Description and abbreviations of the traits analyzed for putative QTL detection, in salinized and non-salinized conditions. Twenty traits involved the accumulation of Na^+ and Cl^- in different plant tissues, and six traits related to growth under salinity and non-salinity conditions (Tozlu et al., in press).

Trait abbreviations	Trait descriptions
STNa	Salinized whole plant Na (mg/g)
STCl	Salinized whole plant Cl (mg/g)
SLfNa	Salinized leaf Na (mg/g)
SLfCl	Salinized leaf Cl (mg/g)
SSrNa	Salinized structure root Na (mg/g)
SSrCl	Salinized structure root Cl (mg/g)
SFrNa	Salinized fine root Na (mg/g)
SFrCl	Salinized fine root Cl (mg/g)
SSmNa	Salinized stem Na (mg/g)
SSmCl	Salinized stem Cl (mg/g)
STCl_Na	Salinized whole plant Cl/Na (mol/mol)
CLfCl	Control leaf Cl (mg/g)
CLfNa	Control leaf Na (mg/g)
CSmCl	Control stem Cl (mg/g)
CSmNa	Control stem Na (mg/g)
CSrCl	Control structure root Cl (mg/g)
CSrNa	Control structure root Na (mg/g)
CTCl	Control whole plant Cl (mg/g)
CTNa	Control whole plant Na (mg/g)
CTCl_Na	Control whole plant Cl/Na (mol/mol)
CNG	Control net growth (cm)
SNG	Salinized net growth (cm)
SNG/CNG	Ratio of SNG to CNG (%)
CTG	Control total growth (cm)
STG	Salinized total growth (cm)
STG/CTG	Ratio of STG to CTG (%)

Table 4-2: The comparisons of molecular marker numbers, map coverage, and marker densities generated by two different mapping studies. The AFLP marker integrated citrus genome map was calculated by JoinMap 2.0 (Stam and Van Ooijen 1995). The previous RAPD-based citrus genome map was generated by MAPMAKER (Lander et al., 1987). The linkage groups designations of the two maps do not corresponded with each other.

AFLP marker integrated citrus linkage map (analyzed by JoinMap)				RAPD-based citrus linkage map (Cai et al. 1994) (analyzed by MAPMAKER)			
Major Linkage groups	Markers[a]	Map length (cM)	Marker density[b]	Major Linkage groups	Markers[a]	Map length (cM)	Marker density[b]
I	85	98.9	1.2	I	37	204.2	5.5
II	55	108.0	2.0	II	35	243.8	7.0
III	180	199.1	1.1	III	20	168.4	8.4
IV	44	99.1	2.2	IV	32	241.9	7.6
V	40	100.1	2.5	V	11	93.4	8.5
VI	38	68.8	1.8	VI	11	105.3	9.6
VII	15	89.3	5.9	VII	7	60.5	8.6
VIII	14	42.2	3.0	VIII	6	29.8	5.0
IX	10	46.6	4.7	IX	3	43.8	14.6
X	9	49.7	5.5	-			
XI	7	63.6	9.1	-			
Total	497	965.4	1.9	Total	162	1191.1	7.3
Minor linkage group				Minor linkage group			
XII	4	21.1	5.3	-	-	-	-
XIII	4	20.9	5.2	-	-	-	-
XIV	2	18.9	9.5	-	-	-	-
XV	2	0.1	-	-	-	-	-
Total	12	61	5.1	-	-	-	-

[a] Number of markers on each linkage group

[b] Average marker densities (cM/marker interval) for each linkage group, and for the entire linkage map.

Table 4-3: The fourteen Na $^+$ and Cl$^-$ accumulation-related traits and their putative QTLs (LOD> 2.2) detected under salinity and non-salinity conditions. The description of the abbreviated trait name was listed in Table 4-1. For each trait, the putative QTLs were indicated by map position and marker loci. The existence of the putative QTLs was determined by LOD scores. The genomic areas with LOD > 2.2 were indicated with bold font. Var. expl. (%) = percentage of phenotypic variance explained by the region; Add. effect = additive effects. The regions with LOD score inconsistency were indicated with "a*sterisk*".

Trait	***Linkage group IV***	***QTL regions***			
ClfNa	Map position	Locus	LOD	Var. expl. (%)	Add. Effect
	23.3	pcPt001	0.54	5.0	-0.230228
	25.0	EACMCTA450	1.35	12.2	-0.343566
	27.0		**2.74**	**30.7**	**-0.545201**
	27.8	**EAAMCAA210**	**2.11**	**18.5**	**-0.423107**
	27.8	**EAAMCAA340**	**2.11**	**18.5**	**-0.423107**
	27.8	ETTMCTC290	0.44	4.1	-0.209193
	29.6	ETGMCTG240	**0.50**	4.7	-0.226692
	Linkage group VI	***QTL regions***			
	Map position	Locus	LOD	Var. expl. (%)	Add. Effect
	28.8	pRLc38	0.10	0.9	-0.099282
	29.9	EAAMCAT302	0.84	7.7	-0.277086
	31.9	EACMCAC215	**0.67**	6.2	-0.253476
	33.9		1.35	12.4	-0.362028
	34.1	**EACTMCAT280**	**1.39**	**12.5**	**-0.363463**
	35.6	**OPG05048C**	**2.20**	**19.0**	**-0.448830**
	36.2	**pgCit045**	**1.71**	**15.2**	**-0.406562**
	36.2	**pgCit042**	**1.71**	**15.2**	**-0.406562**
	36.2	EAAMCTT150	1.38	12.4	-0.362296
Trait	***Linkage group I***	***QTL regions***			
SlfNa	Map position	Locus	LOD	Var. expl (%)	Add. Effect
	28.8	EACAMCTT130	1.50	13.4	2.57695
	29.7	**EACMCTA600**	**2.41**	**20.7**	**3.14071**
	29.7	pRLc32	1.94	17.0	2.86929
	33.7	ETGMCAG220	1.13	10.5	2.32073
Trait	***Linkage group IV***	***QTL regions***			
SlfNa	Map position	Locus	LOD	Var. expl (%)	Add. Effect
	0.0	**OPE19066C**	**2.51**	**21.4**	**3.16461**
	2.0		**2.81**	**26.5**	**3.52911**
	3.2	**pgCit057L**	**2.30**	**19.8**	**3.04455**
	5.2		**3.42**	**35.9**	**4.10942**
	7.2		**4.23**	**41.5**	**4.42091**
	9.2		**4.82**	**44.3**	**4.57162**
	11.2		**5.28**	**45.6**	**4.64816**
	13.2		**5.62**	**45.9**	**4.67830**
	15.2		**5.88**	**45.6**	**4.67532**
	17.2		**6.08**	**44.6**	**4.64862**

(Table 4-3 continued)

Trait	***Linkage group IV***	***QTL regions***			
SlfNa	Map position	Locus	LOD	Var. Expl (%)	Add. effect
	17.6	**pgCit037**	**6.11**	**44.4**	**4.64120**
	18.4	**pRLc66L**	**5.12**	**38.8**	**4.38422**
	18.4	**pgCit039**	**6.11**	**44.4**	**4.64120**
	20.4		**6.43**	**51.2**	**5.01428**
	22.4		**5.04**	**47.6**	**4.85351**
	23.3	**pcPt001**	**3.13**	**25.9**	**3.62850**
	25.0	EACMCTA450	1.57	14.0	2.55277
	27.0		1.38	19.7	3.02410
	27.8	EAAMCAA210	0.61	5.7	1.62801
	27.8	EAAMCAA340	0.61	5.7	1.62801
	27.8	**ETTMCTC290**	**7.55**	**52.1**	**5.13399**
	29.6	**ETGMCTG240**	**6.38**	**46.2**	**4.90474**
	31.6		**6.03**	**45.9**	**4.92751**
	33.2	**EAGMCTA430**	**4.99**	**38.4**	**4.47038**
	35.2		**5.16**	**44.7**	**4.84227**
	37.2		**4.41**	**43.2**	**4.71113**
	39.2		**2.55**	**34.8**	**4.18442**
	39.8	EACMCAA210	1.61	14.3	2.63705
	41.8		**3.78**	**30.6**	**3.82126**
	41.8	**EACAMCAG80**	**3.78**	**30.6**	**3.82126**
	43.8		**4.41**	**40.1**	**4.43546**
	45.8		**3.95**	**37.7**	**4.34457**
	47.8		**3.10**	**31.3**	**4.04455**
	49.2	**OPF14063C**	**2.30**	**20.5**	**3.35120**
	51.2		1.90	20.4	3.37099
	52.0	pgCit074	1.26	11.4	2.47939
Trait	***Linkage group III***	***QTL regions***			
CLfCl	Map position	Locus	LOD	Var. Expl. (%)	Add. effect
	13.7	EAAMCTG80	1.86	16.3	-0.285632
	14.7	**EAGMCAA190**	**3.15**	**26.1**	**-0.355846**
	16.3	**ETTMCTA135**	**5.45**	**40.8**	**-0.458805**
	18.3		**7.06**	**50.7**	**-0.501103**
	19.1	**EAGMCTA180**	**7.08**	**49.3**	**-0.488845**
	19.1	**ETTMCTA195**	**7.71**	**52.3**	**-0.510809**
	19.1	**EACMCAA185**	**7.71**	**52.3**	**-0.510809**
	20.1	**ETCMCTA260**	**6.45**	**46.1**	**-0.467111**
	20.1	**OPG14059C**	**2.50**	**21.3**	**-0.325938**
	20.1	**EAGMCTA185**	**5.83**	**42.9**	**-0.450222**
	22.1		**5.60**	**47.9**	**-0.478589**
	23.1	**EACMCTA295**	**3.83**	**33.9**	**-0.395799**
	24.5	**OPE19142C**	**2.07**	**18.0**	**-0.295633**
	26.2	**ETCMCAC650**	**3.52**	**28.8**	**-0.358806**
	27.5	**EAAMCTT205**	**5.23**	**39.9**	**-0.420695**
	29.5		**4.40**	**39.8**	**-0.420059**
	30.4	**EACTMCAA340**	**3.26**	**27.4**	**-0.350573**
	32.4		1.52	19.0	-0.290075

(Table 4-3 continued)

Trait	***Linkage group III***	***QTL regions***			
CLfCl	Map position	Locus	LOD	Var. Expl. (%)	Add. effect
	131.8	EACMCTA375	1.92	17.3	0.310353
	132.6	pgCit009	**0.08**	0.8	-0.059429
	132.6	**EAGMCTA210***	**3.37**	**27.7**	**0.355143**
	133.4	**ETAMCTA170***	**4.85**	**37.2**	**0.430938**
	133.4	ETAMCTA270	**0.07**	0.7	0.055861
	133.4	pgCit028	**0.01**	0.1	0.016765
	135.1	**EATMCTT285***	**2.66**	**22.5**	**0.318528**
	135.1	**EACMCAC250***	**5.91**	**43.3**	0.464722
	135.7	OPG02092C	**0.34**	3.2	0.120571
	135.7	**EATMCTT250***	**5.91**	**43.3**	0.464688
	135.7	**ETAMCAA230***	**5.10**	**38.7**	0.446788
	136.3	ETGMCAT420	**0.00**	0.0	-0.013140
	136.3	EACMCAC245	**0.40**	3.8	0.130586
	137.7	**ETTMCTG250***	**3.61**	**29.3**	**0.388606**
	137.7	pgCit021	**0.01**	0.1	0.016857
	139.6	ETTMCTG260	**0.03**	0.3	-0.033991
Trait	***Linkage group II***	***QTL regions***			
CSmCl	Map position	Locus	LOD	Var. Expl. (%)	Add. effect
	0.0	EATMCTT160	2.15	19.1	0.152288
	2.0		**2.42**	**23.1**	0.167793
	4.0		**2.47**	**24.4**	**0.172480**
	6.0		2.03	18.0	0.148499
	6.0	EACCMCAA150	2.03	18.0	0.148499
Trait	***Linkage group III***	***QTL regions***			
CSmCl	Map position	Locus	LOD	Var. Expl. (%)	Add. effect
	13.7	EAAMCTG80	1.63	14.5	-0.140749
	14.7	**EAGMCAA190**	**2.55**	**21.7**	**-0.169930**
	16.3	**ETTMCTA135**	**3.36**	**27.6**	**-0.197735**
	18.3		**5.07**	**42.0**	**-0.239146**
	19.1	**EAGMCTA180**	**4.81**	**37.0**	**-0.221780**
	19.1	**ETTMCTA195**	**5.94**	**43.5**	**-0.243958**
	19.1	**EACMCAA185**	**5.94**	**43.5**	**-0.243958**
	20.1	**ETCMCTA260**	**5.09**	**38.6**	**-0.223778**
	20.1	OPG14059C	2.14	18.6	-0.159375
	20.1	**EAGMCTA185**	**4.17**	**33.0**	**-0.206889**
	22.1		**4.08**	**38.8**	**-0.225105**
	23.1	**EACMCTA295**	**2.79**	**24.2**	**-0.175708**
	24.5	OPE19142C	2.19	19.0	-0.158822
	26.2	**ETCMCAC650**	**2.66**	**22.7**	**-0.166658**
	27.5	**EAAMCTT205**	**3.68**	**30.1**	**-0.191349**
	29.5		**3.70**	**34.3**	**-0.204212**
	30.4	**EACTMCAA340**	**3.01**	**25.6**	**-0.177409**
	32.4		1.20	17.4	-0.145368
	131.8	EACMCTA375	1.56	13.9	0.145218
	132.6	pgCit009	0.01	0.1	-0.008143
	132.6	**EAGMCTA210***	**2.33**	**20.0**	**0.158143**

(Table 4-3 continued)

Trait	***Linkage group III***	***QTL regions***			
CSmCl	Map position	Locus	LOD	Var. Expl. (%)	Add. effect
	133.4	**ETAMCTA170***	**3.93**	**31.4**	**0.207188**
	133.4	ETAMCTA270	0.56	5.2	0.079617
	133.4	pgCit028	0.00	0.0	0.003861
	135.1	**EATMCTT285***	**2.81**	**23.6**	**0.170903**
	135.1	**EACMCAC250***	**4.92**	**37.6**	**0.226881**
	135.7	OPG02092C	0.19	1.8	0.047571
	135.7	**EATMCTT250***	**4.92**	**37.6**	**0.226875**
	135.7	**ETAMCAA230***	**5.46**	**40.8**	**0.240121**
	136.3	ETGMCAT420	0.31	2.9	0.060430
	136.3	EACMCAC245	0.89	8.2	0.100637
	137.7	ETTMCTG250	1.54	13.7	0.139273
Trait	***Linkage group I***	***QTL regions***			
STCl	Map position	Locus	LOD	Var. Expl. (%)	Add. effect
	21.1	EAAMCAT180	2.01	17.7	2.02677
	23.1		**2.31**	**20.1**	**2.17000**
	23.5	**OPQ17064C**	**2.36**	**20.3**	**2.18328**
	25.5		1.25	14.0	1.80187
	27.0	EACAMCTT260	0.38	3.6	0.917683
	28.8	**EACAMCTT130**	**2.21**	**19.1**	**2.17035**
	29.7	**EACMCTA600**	**1.83**	**16.1**	**1.95850**
	29.7	**pRLc32**	**3.12**	**25.9**	**2.50347**
	31.7		**2.97**	**28.8**	**2.65175**
	33.7		2.09	19.5	2.23522
	33.7	ETGMCAG220	2.09	19.5	2.23522
	35.3	**EACAMCTT140**	**2.71**	**24.4**	**2.39686**
Trait	***Linkage group II***	***QTL regions***			
STCl	Map position	Locus	LOD	Var. Expl. (%)	Add. effect
	0.0	**EATMCTT160**	**2.91**	**25.3**	**2.41996**
	2.0		**2.82**	**25.0**	**2.40482**
	4.0		**2.71**	**24.2**	**2.36727**
	6.0		**2.55**	**22.5**	**2.28818**
	6.0	**EACCMCAA150**	**2.55**	**22.5**	**2.28818**
Trait	***Linkage group I***	***QTL regions***			
SFrCl	Map position	Locus	LOD	Var. Expl.(%)	Add. effect
	33.7	ETGMCAG220	0.11	1.1	0.587937
	35.3	EACAMCTT140	0.17	1.6	0.697924
	35.3	**EAGMCAA210***	**2.99**	**25.0**	**2.71630**
	37.3		**1.95**	**26.3**	**2.80070**
	39.3		0.13	3.1	0.990670
	40.6	EACMCTG410	0.05	0.5	-0.406830
Trait	***Linkage group IV***	***QTL regions***			
CTCl_ Na	Map position	Locus	LOD	Var. Expl.(%)	Add. Effect
	23.3	pcPt001	0.69	6.4	0.077989
	25.0	EACMCTA450	0.28	2.7	0.048161
	27.0		**2.16***	**23.7**	**0.143428**

(Table 4-3 continued)

	27.8	**EAAMCAA210**	**2.37***	**20.4**	**0.132915**
Trait	***Linkage group IV***	***QTL regions***			
CTCl_ Na	Map position	Locus	LOD	Var. expl.(%)	Add. Effect
	27.8	**EAAMCAA340**	**2.37***	**20.4**	**0.132915**
	27.8	ETTMCTC290	0.55	5.2	0.069937
	29.6	ETGMCTG240	0.85	7.8	0.087394
Trait	***Linkage group I***	***QTL regions***			
SLfCl	Map position	Locus	LOD	Var. expl. (%)	Add. effect
	29.7	EACMCTA600	1.39	12.5	3.90250
	29.7	**pRLc32**	**2.59**	**22.0**	**5.22949**
	31.7		**2.72**	**27.1**	**5.86072**
	33.7		2.15	20.4	5.18728
	33.7	ETGMCAG220	2.15	20.4	5.18728
	35.3	**EACAMCTT140**	**2.43**	**22.3**	**5.19889**
Trait	***Linkage group II***	***QTL regions***			
SLfCl	Map position	Locus	LOD	Var. expl. (%)	Add. effect
	0.0	**EATMCTT160**	**3.74**	**31.3**	**6.09806**
	2.0		**3.46**	**29.8**	**5.95258**
	4.0		**3.12**	**27.6**	**5.72716**
	6.0		**2.74**	**24.0**	**5.34923**
	6.0	**EACCMCAA150**	**2.74**	**24.0**	**5.34923**
Trait	***Linkage group III***	***QTL regions***			
SLfCl	Map position	Locus	LOD	Var. expl. (%)	Add. effect
	16.3	ETTMCTA135	2.13	18.5	-5.06111
	18.3		**3.10**	**26.7**	**-5.92418**
	19.1	**EAGMCTA180**	**3.29**	**27.1**	**-5.92951**
	19.1	**ETTMCTA195**	**2.40**	**20.6**	**-5.24641**
	19.1	**EACMCAA185**	**2.40**	**20.6**	**-5.24641**
	20.1	ETCMCTA260	1.97	17.2	-4.66833
	20.1	OPG14059C	0.72	6.7	-2.98750
	20.1	EAGMCTA185	2.12	18.4	-4.82833
	22.1		2.09	21.2	-5.20600
	23.1	EACMCTA295	1.60	14.4	-4.24659
	24.5	OPE19142C	0.85	7.8	-3.18486
	26.2	ETCMCAC650	1.94	17.2	-4.53824
	27.5	**EAAMCTT205**	**2.60**	**22.5**	**-5.17452**
	29.5		**2.47**	**24.9**	**-5.45304**
	30.4	EACTMCAA340	1.91	17.5	-4.58545
Trait	***Linkage group IX***	***QTL regions***			
SSmCl	Map lod	Locus	LOD	Var. expl. (%)	Add. effect
	29.1	EACTMCAT188	**0.62**	**6.0**	**-0.601365**
	31.1		1.00	12.1	**-0.868811**
	33.1		1.52	20.4	-1.16132
	35.1		2.03	25.0	-1.32879
	37.1		**2.44**	**26.3**	**-1.41016**
	38.2	**ETAMCAA290**	**2.62**	**26.3**	**-1.44079**
	40.2		**2.52**	**29.6**	**-1.48343**

(Table 4-3 continued)

Trait	***Linkage group IX***	***QTL regions***			
SSmCl	Map lod	Locus	LOD	Var. expl. (%)	Add. effect
	44.2		1.32	21.4	-1.24178
	46.2		0.51	5.7	-0.638832
	46.6	EAGMCAA240	0.41	4.1	-0.539006
SSmCl	***Linkage group XIII***	**QTL regions**			
Trait	Map position	Locus	LOD	Var. expl. (%)	Add. effect
	0.0	**OPG06023C**	**3.05**	**25.8**	**1.26193**
	2.0		**3.26**	**29.1**	**1.36023**
	4.0		**3.34**	**30.2**	**1.40274**
	6.0		**3.30**	**30.0**	**1.41111**
	6.1	**OPF15063C**	**3.30**	**30.0**	**1.41077**
	8.1		**2.91**	**29.1**	**1.39749**
	10.1		**2.37**	**26.9**	**1.34682**
	12.1		1.56	19.6	1.14900
	13.2	OPE07070C	1.06	9.7	0.797932
Trait	***Linkage group I***	***QTL regions***			
SSrCl	Map position	Locus	LOD	Var. expl. (%)	Add. effect
	52.7	OPF06750	1.64	14.5	1.75650
	52.7	EAAMCTG50	1.80	15.9	1.78973
	53.4	**RGpt8taqI**	**2.66**	**22.5**	**2.12840**
	54.1	OPAN20340	1.22	11.0	1.49006
	54.6	ETTMCTA170	1.06	9.7	1.43086
	56.5	OPO16650	1.44	12.9	1.70441
	56.5	EACMCTG590	0.96	8.8	1.40716
	57.1	RGPt9Hinf	1.66	14.8	1.65320
	57.1	OPJ07550	1.05	9.5	1.38673
	65.8	ETTMCTC310	0.95	8.8	1.23355
	67.2	OPT08057C	0.87	8.0	1.27297
	67.2	OPO07058C	1.10	10.0	1.42171
	68.2	OPQ09090C	1.36	12.3	1.73165
	68.2	OPA15081C	1.18	10.7	1.56285
	70.2		2.20	25.3	2.19402
	72.2		2.11	19.4	1.87139
	72.4	ETTMCTC580	2.06	18.1	1.80213
	75.7	OPE04051C	1.50	13.4	1.64114
	75.7	**EAAMCAT320**	**2.33**	**20.0**	**1.79979**
	77.7		2.01	23.4	2.04699
	78.9	OPE18087C	1.06	9.6	1.39323
	78.9	EATMCTT140	1.28	11.6	1.37686
SSrCl	***Linkage group III***	***QTL regions***			
Trait	Map position	Locus	LOD	Var. expl. (%)	Add. effect
	0.0	OPF05105C	0.25	2.4	0.615647
	2.0		0.69	13.1	1.44275
	4.0		1.29	21.3	1.84567
	6.0		1.73	24.7	1.99598

(Table 4-3 continued)

SSrCl	***Linkage group III***	***QTL regions***			
Trait	Map position	Locus	LOD	Var. expl. (%)	Add. effect
	8.0		**2.02**	**25.5**	**2.04016**
	10.0		**2.18**	**24.5**	**2.01873**
	12.0		**2.22**	**22.0**	**1.94397**
	13.7	**EAAMCTG80**	**2.20**	**19.0**	**1.84554**
	14.7	**EAGMCAA190**	**2.68**	**22.7**	**1.98493**
	16.3	ETTMCTA135	1.47	13.1	1.55937
	18.3		1.29	12.2	1.47292
	19.1	EAGMCTA180	1.15	10.4	1.34585
	19.1	ETTMCTA195	1.58	14.1	1.58814
	19.1	EACMCAA185	1.58	14.1	1.58814
	20.1	**ETCMCTA260**	**1.90**	**16.7**	**1.68356**
	20.1	**OPG14059C**	**3.05**	**25.4**	**2.13156**
	20.1	EAGMCTA185	1.38	12.4	1.45067
Trait	***Linkage group IV***	***QTL regions***			
SSrNa	Map position	Locus	LOD	Var. expl. (%)	Add. effect
	0.0	**OPE19066C**	**4.89**	**37.5**	**-1.61617**
	2.0		**4.64**	**38.4**	**-1.64398**
	3.2	**pgCit057L**	**3.80**	**30.5**	**-1.45913**
	5.2		**3.99**	**34.8**	**-1.55796**
	7.2		**4.07**	**37.1**	**-1.61314**
	9.2		**4.04**	**38.3**	**-1.64105**
	11.2		**3.90**	**38.4**	**-1.64492**
	13.2		**3.62**	**37.2**	**-1.62074**
	15.2		**3.13**	**33.7**	**-1.54509**
	17.2		**2.37**	**22.1**	**-1.26104**
	17.6	**pgCit037**	**2.21**	**19.1**	**-1.17537**
	18.4	pRLc66L	1.62	14.4	-1.03233
	18.4	**pgCit039**	**2.21**	**19.1**	**-1.17537**
	20.4		2.15	21.4	-1.25516
	22.4		1.62	16.0	-1.09456
	23.3	pcPt001	1.30	11.8	-0.943169
	25.0	EACMCTA450	0.30	2.8	-0.444346
	27.0		0.24	2.8	-0.442407
	27.8	EAAMCAA210	0.15	1.4	-0.311734
	27.8	EAAMCAA340	0.15	1.4	-0.311734
	27.8	**ETTMCTC290**	**3.00**	**25.1**	**-1.37664**
	29.6	**ETGMCTG240**	**2.29**	**19.8**	**-1.24061**
	31.6		1.92	17.7	-1.17539
	33.2	EAGMCTA430	1.49	13.4	-1.02089
Trait	***Linkage group I***	***QTL regions***			
CTCl	Map position	Locus	LOD	Var. expl. (%)	Add. effect
	21.1	EAAMCAT180	1.76	15.7	0.172398
	23.1		**2.39**	**20.8**	**0.199931**
	23.5	**OPQ17064C**	**2.48**	**21.2**	**0.201637**

(Table 4-3 continued)

Trait	***Linkage group I***	***QTL regions***			
CTCl	Map position	Locus	LOD	Var. expl. (%)	Add. effect
	25.5		0.85	11.3	0.146107
	27.0	EACAMCTT260	0.04	0.3	0.025522
	28.8	EACAMCTT130	1.72	15.2	0.175043
	29.7	EACMCTA600	0.56	5.2	0.100929
	29.7	**pRLc32**	**2.56**	**21.8**	**0.207604**
	31.7		**3.47**	**33.7**	**0.258739**
	33.7		**3.21**	**28.4**	**0.241289**
	33.7	**ETGMCAG220**	**3.21**	**28.4**	**0.241289**
	35.3	EACAMCTT140	1.97	18.0	0.185616
Trait	***Linkage group III***	***QTL regions***			
CTCl	Map position	Locus	LOD	Var. expl. (%)	Add. effect
	26.2	ETCMCAC650	1.62	14.5	-0.166125
	27.5	**EAAMCTT205**	**2.42**	**21.0**	**-0.199479**
	29.5		**2.28**	**22.0**	**-0.204385**
	30.4	EACTMCAA340	1.93	17.4	-0.182371
Trait	***Linkage group I***	***QTL regions***			
STNa	Map position	Locus	LOD	Var. expl. (%)	Add. effect
	18.7	EACAMCTT270	0.68	6.3	0.662968
	20.7		**2.64**	**23.8**	**1.28835**
	21.1	**EAAMCAT180**	**2.89**	**24.3**	**1.30093**
	23.1		**2.62**	**22.3**	**1.25349**
	23.5	**OPQ17064C**	**2.55**	**21.7**	**1.23759**
	25.5		1.58	17.5	1.10270
	27.0	EACAMCTT260	0.60	5.6	0.626063
	28.8	EACAMCTT130	1.96	17.1	1.12740
	29.7	**EACMCTA600**	**2.82**	**23.7**	**1.30293**
	29.7	**pRLc32**	**2.63**	**22.3**	**1.27381**
	31.7		**2.20**	**20.4**	**1.22232**
	33.7		1.51	13.6	1.02156
	33.7	ETGMCAG220	1.51	13.6	1.02156
Trait	***Linkage group IV***	***QTL regions***			
STNa	Map position	Locus	LOD	Var. expl. (%)	Add. effect
	17.6	pgCit037	2.03	17.7	1.13356
	18.4	pRLc66L	1.66	14.7	1.04389
	18.4	pgCit039	2.03	17.7	1.13356
	20.4		1.90	18.9	1.18017
	22.4		1.44	15.5	1.08200
	23.3	pcPt001	1.07	9.8	0.861195
	25.0	EACMCTA450	0.89	8.1	0.753515
	27.0		0.81	12.2	0.920800
	27.8	EAAMCAA210	0.37	3.5	0.493972
	27.8	EAAMCAA340	0.37	3.5	0.493972
	27.8	**ETTMCTC290**	**2.49**	**21.2**	**1.27056**
	29.6	**ETGMCTG240**	**2.08**	**18.1**	**1.18882**
	31.6		**2.13**	**19.0**	**1.22445**
	33.2	**EAGMCTA430**	**2.08**	**18.1**	**1.18895**

(Table 4-3 continued)

Trait	***Linkage group II***	***QTL regions***			
STCl_Na	Map position	locus	LOD	Var. expl. (%)	Add. effect
	16.1	EAAMCAA250	0.43	4.7	0.125774
	16.1	EAAMCAA430	0.43	4.7	0.125774
	17.7	ETAMCTG265	1.07	11.0	0.194766
	19.4	ETGMCTA190	0.74	7.8	0.158385
	21.4		2.05	30.3	0.307308
	23.4		**2.30**	**25.5**	**0.281878**
	24.3	**EAGMCAA660**	**2.21**	**21.0**	**0.259089**
STCl_Na	***Linkage group IV***	***QTL regions***			
	Map position	Locus	LOD	Var. expl. (%)	Add. effect
	0.0	OPE19066C	0.41	3.9	-0.110056
	2.0		0.26	2.8	-0.092654
	3.2	pgCit057L	0.14	1.4	-0.065315
	5.2		0.31	3.4	-0.103209
	7.2		0.57	6.7	-0.145531
	9.2		0.91	10.7	-0.183908
	11.2		1.28	14.3	-0.213201
	13.2		1.66	17.1	-0.234037
	15.2		2.03	19.2	-0.248560
	17.2		**2.38**	**20.7**	**-0.258542**
	17.6	**pgCit037**	**2.45**	**20.9**	**-0.260109**
	18.4	**pRLc66L**	**2.68**	**22.6**	**-0.273333**
	18.4	**pgCit039**	**2.45**	**20.9**	**-0.260109**
	20.4		**3.08**	**29.3**	**-0.312489**
	22.4		**2.92**	**27.4**	**-0.304491**
	23.3	**pcPt001**	**2.59**	**22.0**	**-0.272713**
	25.0	EACMCTA450	1.18	10.7	-0.182246
	27.0		1.88	18.7	-0.240706
	27.8	EAAMCAA210	1.85	16.3	-0.224845
	27.8	EAAMCAA340	1.85	16.3	-0.224845
	27.8	**ETTMCTC290**	**2.46**	**21.1**	**-0.267003**
	29.6	**ETGMCTG240**	**2.72**	**23.1**	**-0.283381**
	31.6		**2.44**	**22.0**	**-0.278072**
	33.2	EAGMCTA430	1.99	17.5	-0.246784
	35.2		2.04	20.4	-0.263396
	37.2		1.82	19.1	-0.251312
	39.2		1.40	13.6	-0.210139
	39.8	EACMCAA210	1.25	11.4	-0.191882
	41.8		**2.43**	**20.9**	**-0.257897**
	41.8	**EACAMCAG80**	**2.43**	**20.9**	**-0.257897**
	43.8		**2.45**	**23.2**	**-0.276507**
	45.8		**2.31**	**22.9**	**-0.280279**
	47.8		1.99	20.7	-0.272678
	49.2	OPF14063C	1.59	14.6	-0.231580
	51.2		1.55	16.9	-0.247409
	52.0	pgCit074	1.14	10.4	-0.193212
	52.0	ETCMCAC370	1.35	12.2	-0.198258

Table 4-4: The six growth-related traits and their putative QTLs (LOD> 2.2) detected under salinity and non-salinity conditions. The description of the abbreviated trait name was listed in Table 4-1. For each trait, the putative QTLs were indicated by map position and marker loci. The existence of the putative QTLs was determined by LOD scores. The genomic areas with LOD > 2.2 were indicated with bold font. Var. expl (%) = percentage of phenotypic variance explained by the region; Add. effect = additive effects. The regions with LOD score inconsistency were indicated with "*asterisk*".

Traits	***Linkage V***	***QTL regions***			
CTG	Map position	Locus	LOD	Var. expl.(%)	Add. effects
	46.0	ETAMCTT190	1.87	16.6	38.9962
	46.8	**ETGMCAT190**	**3.11**	**26.1**	**48.6750**
	47.8		**3.52**	**32.7**	**54.5353**
	48.5	**ETAMCTT230**	**2.92**	**24.9**	**47.4672**
	49.5		**3.21**	**28.0**	**50.1795**
	50.1	**EACTMCTC280**	**3.17**	**26.7**	**49.0946**
	50.6	**EACMCTC380**	**2.90**	**24.8**	**47.2126**
	50.6	**ETGMCAT100**	**2.90**	**24.8**	**47.2126**
	51.6		**3.45**	**29.9**	**51.9240**
	52.6		**3.75**	**31.2**	**53.0546**
	52.9	**EACMCTA370**	**3.82**	**31.4**	**53.2221**
	53.9		**3.98**	**34.3**	**55.5543**
	54.9		**3.70**	**31.4**	**53.4302**
	55.0	**EAGMCAC170**	**3.63**	**30.3**	**52.5846**
	56.0		**3.55**	**30.3**	**52.6260**
	56.9	**EAGMCAA185**	**3.37**	**28.5**	**51.0096**
	57.9		**2.77**	**24.7**	**47.3393**
	58.0	**ETAMCAT310**	**2.65**	**23.2**	**45.7768**
	59.0		**2.58**	**22.6**	**45.1072**
	59.3	**EACAMCTT180**	**2.56**	**22.4**	**44.9022**
	60.3		1.94	18.2	40.4593
	60.4	ETAMCTA520	1.82	16.4	38.4612
	61.4		**2.70**	**25.2**	**47.6913**
	62.3	**ETTMCTG265**	**3.11**	**26.4**	**48.8777**
	63.3		**2.75**	**25.7**	**48.1183**
	64.3		**2.23**	**23.2**	**45.7128**
	65.3		1.47	16.2	38.1749
	91.6	OPF05088C	0.56	5.2	22.6936
	93.6		1.22	14.8	38.3552
	94.6		1.60	19.0	43.6404
	95.6		1.95	22.0	47.0463
	96.6		**2.28**	**23.9**	**49.2730**
	97.6		**2.56**	**25.2**	**50.7580**
	98.6		**2.81**	**26.0**	**51.7391**
	99.6		**3.02**	**26.4**	**52.3414**
	100.1	**ETAMCAT410**	**3.12**	**26.5**	**52.5202**

(Table 4-4 continued)

Traits	***Linkage VI***	***QTL regions***			
CTG	Map position	locus	LOD	Var. expl(%)	Add. effect
	4.7	OPQ06089C	1.86	16.4	38.6252
	5.7		2.14	21.6	44.3597
	6.7		**2.35**	**24.7**	**47.3726**
	7.7		**2.49**	**26.2**	**48.7009**
	8.7		**2.57**	**26.9**	**49.2852**
	9.7		**2.59**	**27.1**	**49.4627**
	10.7		**2.56**	**27.0**	**49.3116**
	11.7		**2.48**	**26.4**	**48.7408**
	12.7		**2.32**	**24.9**	**47.3046**
	13.7		2.02	20.4	42.8559
	14.2	OPT06090C	1.83	16.1	38.1162
Traits	***Linkage V***	***QTL regions***			
CNG	Map position	Locus	LOD	Var. Expl. (%)	Add. effects
	42.6	EAAMCAT760	1.56	14.0	23.7915
	43.6		2.15	21.5	29.1265
	44.6		**2.61**	**25.6**	**31.7042**
	45.6		**2.83**	**25.8**	**32.0002**
	46.0	**ETAMCTT190**	**2.81**	**24.0**	**30.9705**
	46.8	**ETGMCAT190**	**3.18**	**26.8**	**32.5498**
	47.8		**4.44**	**38.9**	**39.3403**
	48.5	**ETAMCTT230**	**4.26**	**34.2**	**36.7919**
	49.5		**4.25**	**35.9**	**37.5721**
	50.1	**EACTMCTC280**	**3.81**	**31.4**	**35.1363**
	50.6	**EACMCTC380**	**3.67**	**30.4**	**34.5737**
	50.6	**ETGMCAT100**	**3.67**	**30.4**	**34.5737**
	51.6		**3.89**	**32.9**	**36.0075**
	52.6		**3.90**	**32.6**	**35.8557**
	52.9	**EACMCTA370**	**3.87**	**31.9**	**35.4620**
	53.9		**3.67**	**31.3**	**35.0691**
	54.9		**3.39**	**29.0**	**33.9521**
	55.0	**EAGMCAC170**	**3.35**	**28.6**	**33.7662**
	56.0		**3.64**	**31.0**	**35.1205**
	56.9	**EAGMCAA185**	**3.83**	**32.1**	**35.7750**
	57.9		**3.48**	**29.8**	**34.3143**
	58.0	**ETAMCAT310**	**3.43**	**29.3**	**34.0146**
	59.0		**3.50**	**29.8**	**34.2491**
	59.3	**EACAMCTT180**	**3.51**	**29.9**	**34.2954**
	60.3		**2.89**	**25.9**	**31.9132**
	60.4	**ETAMCTA520**	**2.76**	**24.2**	**30.8552**
	61.4		**3.41**	**30.3**	**34.5326**
	62.3	**ETTMCTG265**	**3.72**	**31.4**	**35.1774**
	63.3		**3.30**	**30.5**	**34.6535**
	64.3		**2.70**	**27.8**	**33.0335**
	65.3		1.78	19.8	27.8666

(Table 4-4 continued)

Traits	***Linkage V***	***QTL regions***			
CNG	Map position	Locus	LOD	Var. Expl. (%)	Add. effects
	65.8	OPO14090C	1.24	11.3	21.0759
	66.8		2.12	23.7	30.5077
	67.8		**2.29**	**22.4**	**29.8941**
	68.4	EAGMCAG220	2.19	19.0	27.9436
	69.4		2.08	20.0	28.4676
	91.6	OPF05088C	0.50	4.7	14.1970
	92.6		0.77	8.7	19.3083
	93.6		1.11	13.7	24.3217
	94.6		1.47	17.9	27.8792
	95.6		1.81	20.8	30.1551
	96.6		2.12	22.8	31.6452
	97.6		**2.39**	**24.1**	**32.6493**
	98.6		**2.63**	**24.9**	**33.3254**
	99.6		**2.83**	**25.2**	**33.7541**
	100.1	**ETAMCAT410**	**2.92**	**25.3**	**33.8886**
Traits	***Linkage VI***	***QTL regions***			
CNG	Map position	Locus	LOD	Var. Expl(%)	Add. effects
	4.7	**OPQ06089C**	**2.40**	**20.6**	**28.6536**
	5.7		**2.45**	**22.3**	**29.7655**
	6.7		**2.46**	**23.4**	**30.4731**
	7.7		**2.44**	**24.1**	**30.8622**
	8.7		**2.39**	**24.4**	**31.0024**
	9.7		**2.30**	**24.3**	**30.9231**
	10.7		2.16	23.8	30.5970
	11.7		1.97	22.7	29.8765
	12.7		1.71	20.3	28.2163
	13.7		1.35	14.3	23.7308
	14.2	OPT06090C	1.17	10.7	20.4622
Traits	***Linkage V***	***QTL regions***			
STG	Map position	Locus	LOD	Var. expl.(%)	Add. effects
	50.6	ETGMCAT100	1.40	13.5	26.3517
	51.6		2.02	20.5	32.5168
	52.6		**2.44**	**22.8**	**34.3394**
	52.9	**EACMCTA370**	**2.53**	**23.2**	**34.6273**
	53.9		**2.60**	**26.0**	**36.6515**
	54.9		2.17	21.0	33.1157
Traits	***Linkage VI***	***QTL regions***			
STG	Map position	Locus	LOD	Var. expl.(%)	Add. effects
	0.0	ETAMCTG270	0.13	1.3	8.72633
	1.0		0.58	7.4	20.2440
	2.0		1.38	17.4	30.5826
	3.0		**2.30**	**24.7**	**36.1736**
	4.0		**3.17**	**29.2**	**39.1347**
	Linkage VI	***QTL regions***			
STG	Map position	Locus	LOD	Var, expl.(%)	Add. effects

(Table 4-4 continued)

	4.7	**OPQ06089C**	**3.73**	**31.2**	**40.4320**
	5.7		**3.92**	**35.4**	**43.1741**
	6.7		**3.96**	**36.4**	**43.7126**
	7.7		**3.92**	**36.4**	**43.6494**
	8.7		**3.84**	**35.9**	**43.3314**
	9.7		**3.72**	**35.3**	**42.8503**
	10.7		**3.56**	**34.4**	**42.2331**
	11.7		**3.37**	**33.2**	**41.4624**
	12.7		**3.10**	**31.6**	**40.3843**
	13.7		**2.69**	**27.5**	**37.6570**
	14.2	**OPT06090C**	**2.39**	**21.5**	**33.2804**
	15.2		**2.92**	**30.0**	**39.3297**
	16.2		**2.83**	**29.2**	**38.8058**
	17.2		**2.46**	**25.8**	**36.5357**
	18.0	EACMCAC200	1.91	17.5	30.0304
Traits	***Linkage VIII***	***QTL regions***			
STG	Map position	Locus	LOD	Var. expl.(%)	Add. effects
	21.6	**pRLc91**	**2.25**	**20.2**	**35.0324**
	22.6		1.97	18.6	33.7626
	23.6		1.64	16.1	31.5310
	24.6		1.26	12.0	27.5758
	24.7	OPA11059C	1.22	11.5	27.0653
	25.7		1.42	13.4	29.7988
	26.0	OPG10131C	1.47	13.7	30.2708
	26.8	EACCMCAA595	1.47	14.2	30.7749
	27.8		1.77	18.8	36.1560
	28.8		1.85	19.0	36.3181
	29.8		1.73	15.9	32.6514
	29.8	pgCit030	1.73	15.9	32.6514
	30.8		2.07	19.7	36.6673
	31.8		**2.24**	**20.4**	**36.9347**
	32.0	**EACMCTC150**	**2.26**	**20.4**	**36.8628**
	33.0		2.07	19.2	36.3971
	34.0		1.81	16.9	35.3873
	34.1	EACCMCAC370	1.78	16.6	35.2329
	41.0		1.86	19.7	38.0906
	42.0		**2.24**	**21.1**	**39.3875**
	42.2	**ETAMCTG340**	**2.31**	**21.2**	**39.5401**
Traits	***Linkage I***	***QTL regions***			
SNG	Map position	Locus	LOD	Var. expl.(%)	Add. effects
	28.8	**EACAMCTT130**	**3.29**	**28.1**	**-24.3664**
	29.7	**EACMCTA600**	**2.41**	**21.5**	**-20.9820**
	29.7	**pRLc32**	**2.34**	**20.9**	**-20.8358**
	30.7		**2.95**	**30.0**	**-25.3236**
	31.7		**3.20**	**31.9**	**-26.2340**
	32.7		**3.19**	**32.1**	**-26.3495**

(Table 4-4 continued)

Traits	***Linkage I***	***QTL regions***			
SNG	Map position	Locus	LOD	Var. expl.(%)	Add. effects
	33.7	**ETGMCAG220**	**2.61**	**23.6**	**-22.9268**
	34.7		**3.72**	**34.9**	**-27.0392**
	35.3	**EACAMCTT140**	**3.50**	**30.5**	**-25.0253**
Traits	***Linkage V***	***QTL regions***			
SNG	Map position	Locus	LOD	Var. expl.(%)	Add. effects
	46.8	**ETGMCAT190**	**2.54**	**23.1**	**21.6718**
	47.8		**2.34**	**25.1**	**22.5490**
	48.5	ETAMCTT230	1.50	14.6	17.1928
	49.5		1.60	16.0	17.9316
	50.1	EACTMCTC280	1.61	15.7	17.7912
	50.6	EACMCTC380	1.66	16.1	18.0379
	50.6	ETGMCAT100	1.66	16.1	18.0379
	51.6		**2.21**	**22.2**	**21.1883**
	52.6		**2.59**	**24.6**	**22.2755**
	52.9	**EACMCTA370**	**2.68**	**25.0**	**22.4545**
	53.9		**2.40**	**24.6**	**22.2795**
	54.9		1.81	18.3	19.3468
	59.0		1.96	19.9	20.0415
	59.3	EACAMCTT180	2.01	20.2	20.1812
	60.3		**2.34**	**23.2**	**21.6449**
	60.4	**ETAMCTA520**	**2.36**	**23.3**	**21.6883**
	61.4		2.11	21.5	20.8436
	62.3	ETTMCTG265	1.82	18.4	19.3211
	63.3		1.42	15.6	17.7633
Traits	***Linkage VI***	***QTL regions***			
SNG	Map position	Locus	LOD	Var. expl.(%)	Add. effects
	2.0		**2.42**	**33.8**	**26.7325**
	3.0		**3.75**	**39.1**	**28.5161**
	4.0		**4.81**	**41.4**	**29.2042**
	4.7	**OPQ06089C**	**5.42**	**42.3**	**29.4509**
	5.7		**5.25**	**42.5**	**29.5240**
	6.7		**5.05**	**42.4**	**29.5281**
	7.7		**4.82**	**42.2**	**29.4537**
	8.7		**4.55**	**41.8**	**29.2821**
	9.7		**4.23**	**40.9**	**28.9761**
	10.7		**3.86**	**39.6**	**28.4597**
	11.7		**3.40**	**37.3**	**27.5591**
	12.7		**2.84**	**32.9**	**25.8070**
	14.2	OPT06090C	1.86	17.4	18.7593
Traits	***Linkage VI***	***QTL regions***			
SNG	Map position	Locus	LOD	Var. expl.(%)	Add. effects
	15.2		**2.35**	**25.4**	**22.6363**
	16.2		**2.41**	**25.1**	**22.4901**
	17.2		**2.26**	**22.6**	**21.3594**
	18.0	EACMCAC200	2.02	18.3	19.2179

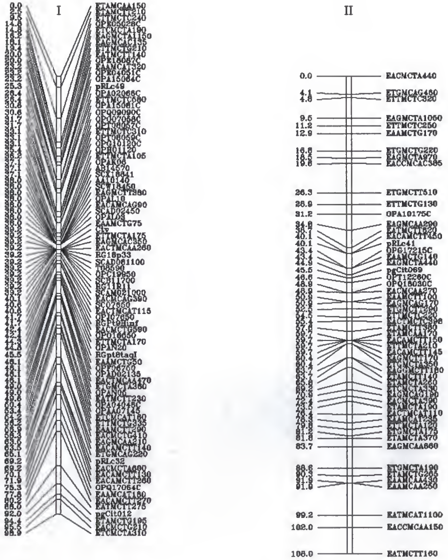
(Table 4-4 continued)

Traits	***Linkage I***	***QTL regions***			
STG/CTG	Map position	Locus	LOD	Var. expl. (%)	Add. effect
	23.5	OPQ17064C	1.75	16.1	-0.073913
	24.5		2.09	22.8	-0.087620
	25.5		**2.21**	**25.8**	**-0.093754**
	26.5		1.85	22.8	-0.088695
	27.0	EACAMCTT260	1.40	13.1	-0.067381
	28.0		**2.44**	**26.9**	**-0.095255**
	28.8	EACAMCTT130	2.19	19.7	-0.083450
	29.7	EACMCTA600	1.55	14.3	-0.070115
	29.7	pRLc32	2.17	19.5	-0.082359
	30.7		**2.25**	**21.4**	**-0.086884**
	31.7		**2.26**	**21.9**	**-0.088529**
	32.7		2.17	21.3	-0.087829
	33.7		1.94	17.8	-0.081443
	33.7	ETGMCAG220	1.94	17.8	-0.081443
	34.7		2.11	21.0	-0.085991
	35.3	EACAMCTT140	1.87	17.2	-0.077001
	40.6	EACMCTG410	1.29	12.2	-0.069157
	41.6		**2.11**	**20.7**	**-0.093051**
	42.0	**EAAMCTT290**	**2.24**	**20.2**	**-0.093170**
Trait	***Linkage VI***	***QTL regions***			
STG/CTG	Map position	Locus	LOD	Var. expl. (%)	Add. effect
	52.6	OPO06058C	0.00	0.0	0.003722
	53.6		0.00	0.0	0.002407
	54.6		0.00	0.0	-0.000147
	55.2	EATMCTC145	0.00	0.0	-0.001616
	55.2	**ETTMCTC150***	**3.14**	**29.4**	0.111484
	56.2		2.12	26.4	0.106308
	57.2		0.82	14.7	0.079786
	58.2		0.03	0.4	0.013017
	58.6	ETAMCAT160	0.00	0.0	-0.003761
	59.2	ETCMCAT80	0.01	0.1	0.007796
Traits	***Linkage I***	***QTL regions***			
SNG/CNG	Map position	Locus	LOD	Var. expl. (%)	Add. Effects
	21.1	EAAMCAT180	2.18	19.7	-0.123737
	22.1		**2.32**	**21.5**	**-0.129440**
	23.1		**2.35**	**21.6**	**-0.129764**
	23.5	**OPQ17064C**	**2.34**	**21.3**	**-0.129153**
	24.5		**2.32**	**23.9**	**-0.136525**
	25.5		2.03	21.9	-0.130631
	26.5		1.57	16.3	-0.113416
	27.0	EACAMCTT260	1.31	12.3	-0.099190
	28.0		**2.73**	**27.0**	**-0.145418**

(Table 4-4 continued)

	28.8	**EACAMCTT130**	**3.64**	**30.6**	**-0.157974**
	29.7	EACMCTA600	1.97	17.9	-0.119115
Traits	***Linkage I***	***QTL regions***			
SNG/CNG	Map position	Locus	LOD	Var. expl. (%)	Add. Effects
	30.7		**3.34**	**32.6**	**-0.163937**
	31.7		**3.60**	**34.4**	**-0.169114**
	32.7		**3.61**	**34.4**	**-0.169516**
	33.7		**3.13**	**27.3**	**-0.153059**
	33.7	**ETGMCAG220**	**3.13**	**27.3**	**-0.153059**
	34.7		**3.93**	**36.4**	**-0.171940**
	35.3	**EACAMCTT140**	**3.53**	**30.4**	**-0.155199**
Traits	***Linkage VI***	***QTL regions***			
SNG/CNG	Map position	Locus	LOD	Var. expl. (%)	Add. Effects
	3.0		1.66	21.0	0.130590
	4.0		2.20	22.3	0.133473
	4.7	**OPQ06089C**	**2.49**	**22.4**	**0.133050**
	5.7		**2.30**	**21.7**	**0.130863**
	6.7		2.10	20.8	0.128077
	7.7		1.89	19.7	0.124483
	8.7		1.66	18.2	0.119762
	9.7		1.43	16.4	0.113436
	10.7		1.18	14.0	0.104872
	11.7		0.94	11.2	0.093566
	12.7		0.72	8.2	0.079945
	13.7		0.53	5.5	0.065681
	14.2	OPT06090C	0.44	4.5	0.058867

Figure 4-1(AB): A. The integrated citrus genome map generated by combining various molecular marker types from two different mapping experiments, using the same intergeneric BC1 family. Minor linkage groups were indicated with "*asterisk*". B. The RAPD marker-based citrus genome map (Cai et al., 1994). The genomic gap regions lost in the integrated mapping processes were indicated with arrow heads.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OPE04051C
OPA15064C
pRLc49
OPA02066C
ETTMCTC580
OPA15081C
OPQ09090C
OPO07058C
OPT08057C
ETTMCTC310
OPT08059C
OPG10120C
OPH01120
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AB14570
SCX18641
AA10140
SCW18450
EAGMCTT380
OPAL10
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SCAD02450
OPAL03
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Ctv
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EAGMCAC350
EACTMCAA260
RG18p33
SCAD081100
T08590
OPC19850
SCB11700
RG11R11
SCAM021000
EACMCAG390
SC07650
EACTMCAT115
OPI07650
RGPt9Hinf
EACMCTG590
OPO16650
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OPAN20
RGpt8taqI
EAAMCTG50
OPF06760
OPAD02135
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ETGMCTA360
OPAN06
EATMCTT230
OPA04046C
OPAA07145
ETCMCAT160
ETTMCTG335
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EACMCTG410
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EACAMCTT140
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pRLc32
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EACAMCTT130
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EAAMCAT180
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EATMCTT275
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EACMCTG210
ETCMCTA310
0.0
2.5
9.5
14.6
25.3
45.5
46.1
66.1
69.2
69.2
70.1
71.9
75.3
77.6
80.2
86.0
92.0
94.4
95.5
98.9
II
0.0 EACMCTA440
4.1 ETGMCAG480
4.8 ETTMCTC320
9.5 EAGMCTA1050
11.2 ETTMCTC250
12.9 EAAMCTG170
16.8 ETGMCTG220
18.5 EAGMCTA970
19.8 EACCMCAC385
26.3 ETGMCTT510
28.9 ETTMCTG130
31.2 OPA10175C
34.9 EAGMCAA290
35.7 EATMCTT620
40.1 EACAMCTT450
40.1 pRLc41
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43.4 EAAMCTG140
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45.5 pgCit069
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50.9 EAAMCTT100
50.9 EAGMCAG170
52.5 ETGMCTT290
54.5 ETTMCTC220
55.4 EACTMCTC398
57.6 ETAMCTT380
59.7 ETAMCAA170
59.7 EACAMCTT150
59.7 ETTMCTA210
59.7 EACAMCTT145
60.4 EAGMCTT170
60.4 ETGMCTA350
63.3 EAGGMCTT180
63.7 ETAMCTT140
65.8 ETAMCTA260
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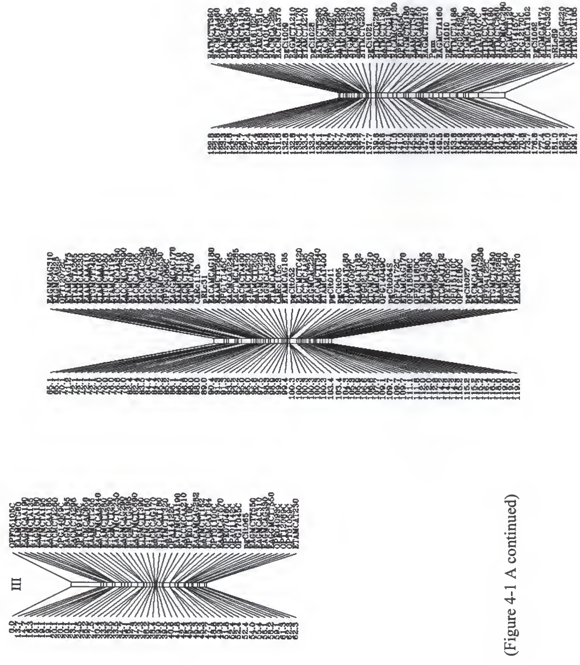
Figure 4-1 A



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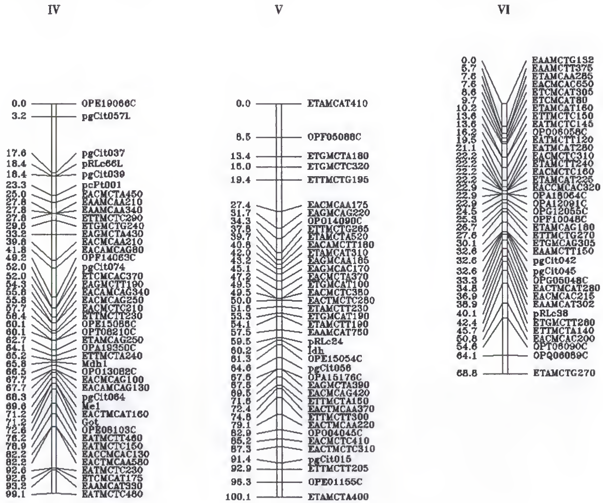

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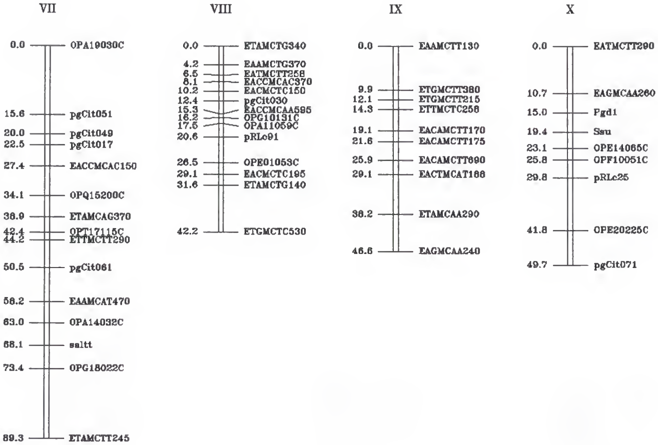

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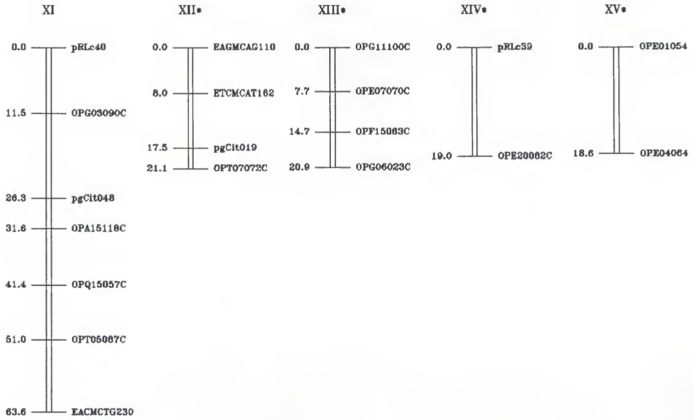

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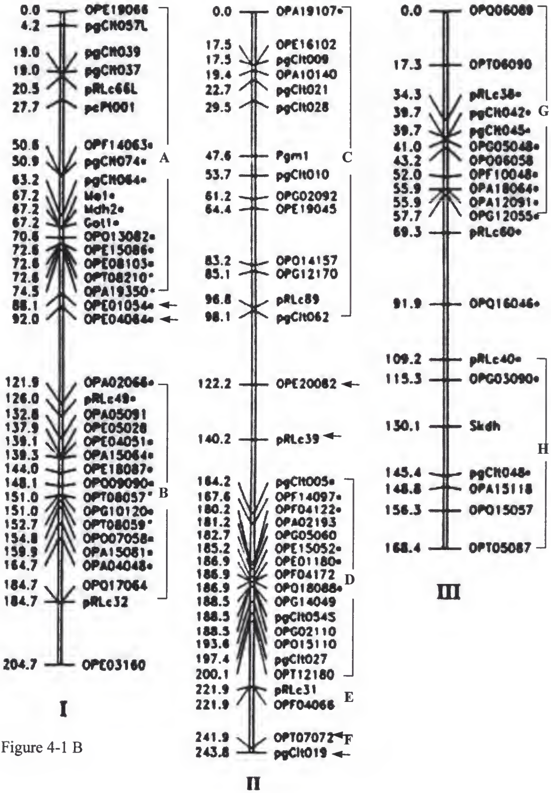
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19.0 pgCit037
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27.7 pcPt001
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63.2 pgCit064•
67.2 Me1•
67.2 Mdh2•
67.2 Got1•
70.6 OPO13082•
72.6 OPE15086•
72.6 OPE08103•
72.6 OPT08210*
74.5 OPA19350*
88.1 OPE01054•
92.0 OPE04084•
121.9 OPA02066•
126.0 pRLc49•
132.8 OPA05091
137.9 OPE05028
139.1 OPE04051•
139.3 OPA15064•
144.0 OPE18087•
148.1 OPO09090•
151.0 OPT08057*
151.0 OPG10120•
152.7 OPT08059*
154.8 OPO07058•
159.9 OPA15081•
164.7 OPA04048•
184.7 OPO17064
184.7 pRLc32
204.7 OPE03160
A
B
I
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17.5 pgCit009
19.4 OPA10140
22.7 pgCit021
29.5 pgCit028
47.6 Pgm1
53.7 pgCit010
61.2 OPG02092
64.4 OPE19045
83.2 OPO14157
85.1 OPG12170
96.8 pRLc89
98.1 pgCit062
122.2 OPE20082
140.2 pRLc39
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167.6 OPF14097•
180.2 OPF04122•
181.2 OPA02193
182.7 OPG05060
185.2 OPE15052•
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188.3 OPG02110
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D
E
F
II
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91.9 OPQ16046•
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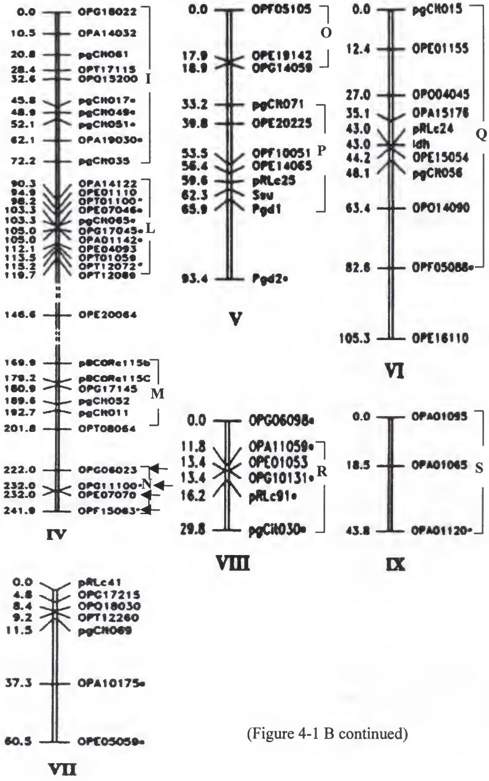
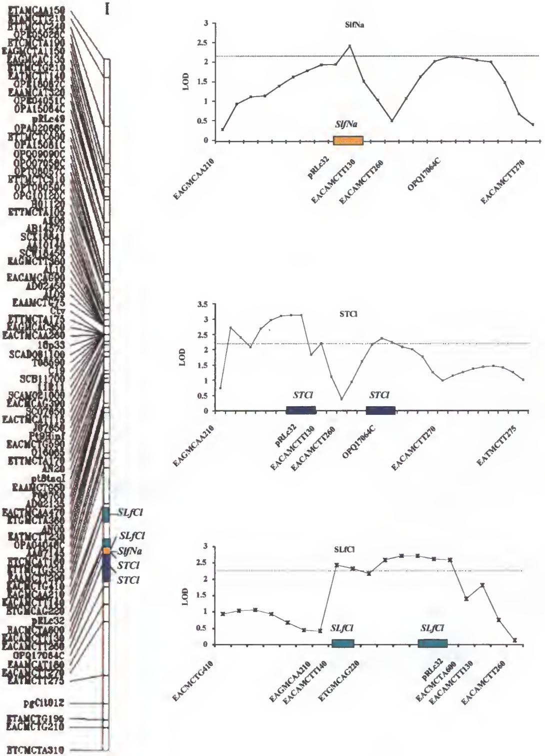
G
H
III

Figure 4-1 B



(Figure 4-1 B continued)

Figure 4-2. The map position of putative QTLs for 6 growth-related traits and 14 salt tolerance related traits. The putative QTL LOD threshold level (2.2) was indicated with a dashed line in the graph. The genomic regions for the corresponding LOD peaks were indicated by shading and indicated with the abbreviated trait names (in Italic). The linkage groups were generated with integrated AFLPs and other types of molecular markers.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OPA15061C
OPQ09090C
OPQ07058C
OPT08059C
OPG10120C
ETTMCTA105
AB14570
EAGMCTT360
EACAMCAG90
EAAMCTC75
Ctv
EACTMCAA260
18p33
SCAD081100
SCB11700
EACTMCAT115
Pt9HinI
ETTMCTA170
pt8taqI
EAAMCTG50
EACTMCAA470
EATMCTT230
pRLc32
EACMCTA600
EACAMCTT130
EACAMCTT260
OPQ17064C
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ETCMCTA310
SLfCl
SLfCl
SlfNa
STCl
STCl
SlfNa
LOD
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2.5
2
1.5
1
0.5
0
SlfNa
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OPQ17064C
EACAMCTT270
STCl
LOD
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3
2.5
2
1.5
1
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STCl
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SLfCl
LOD
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2
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1
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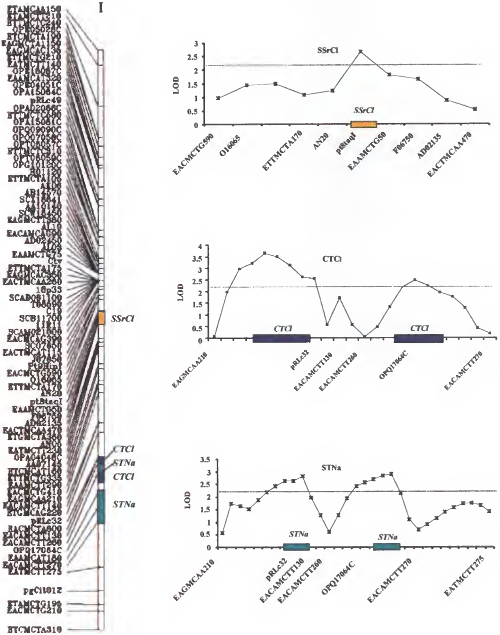
Figure 4-2



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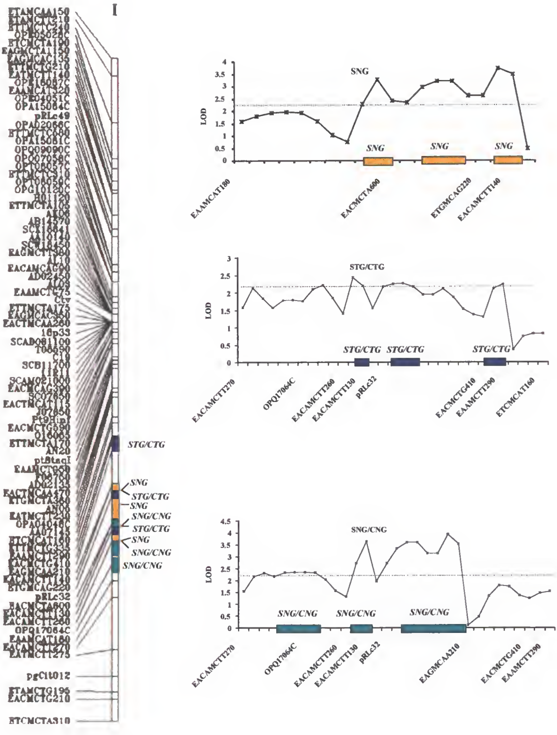

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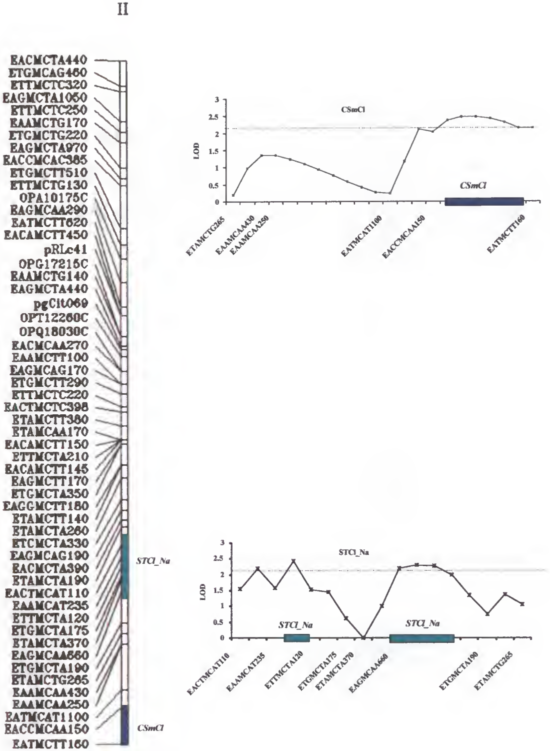

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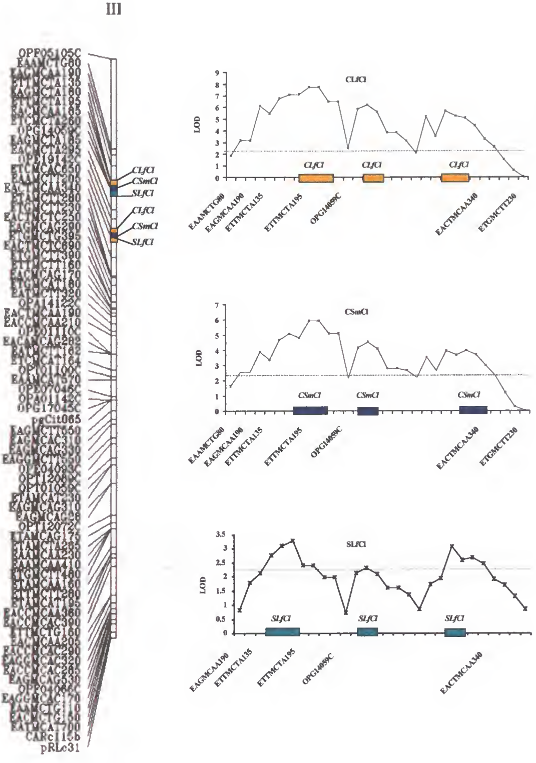

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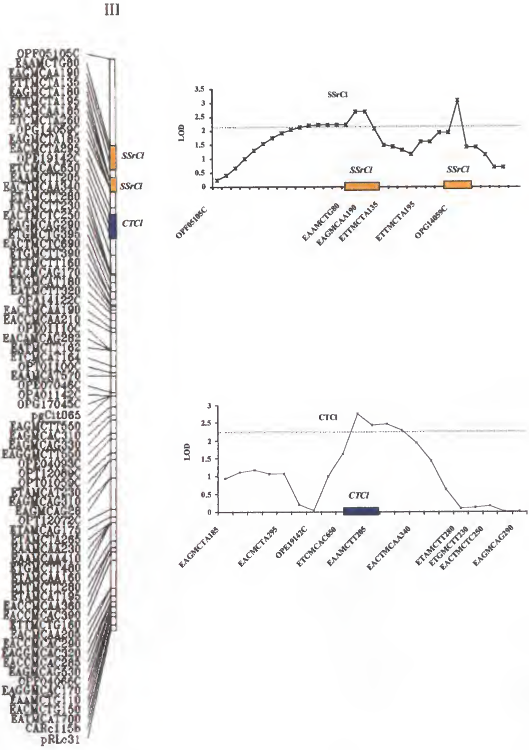

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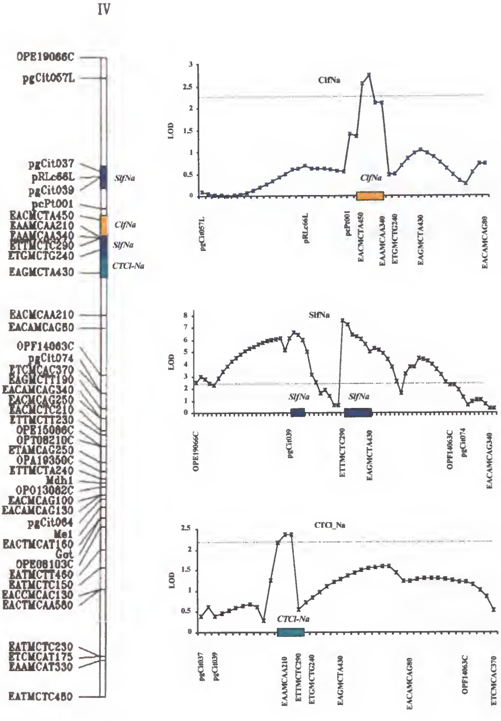

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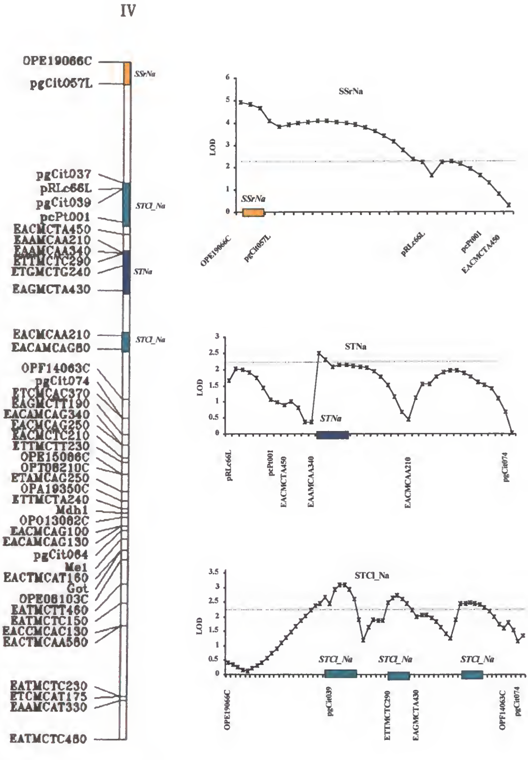

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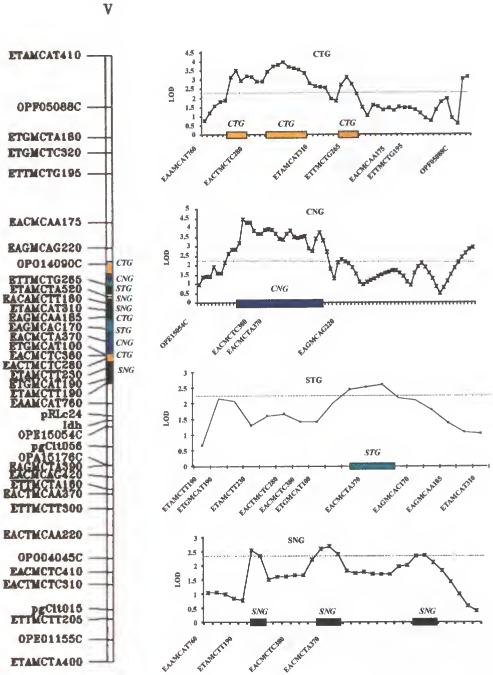

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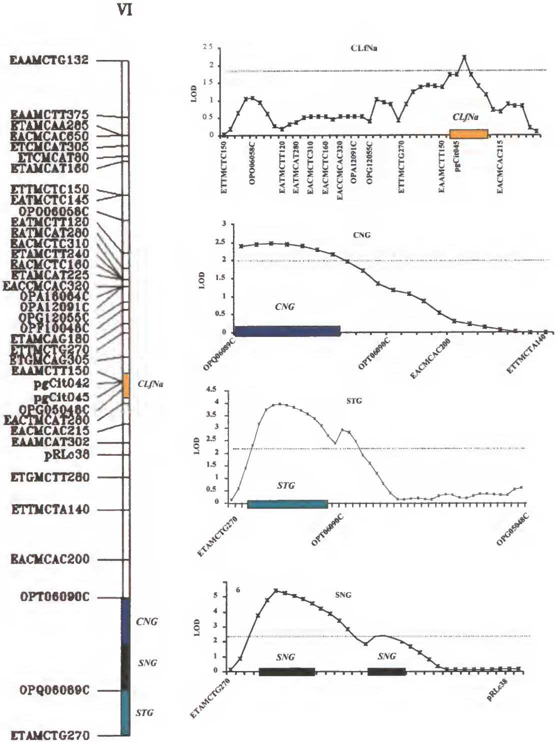

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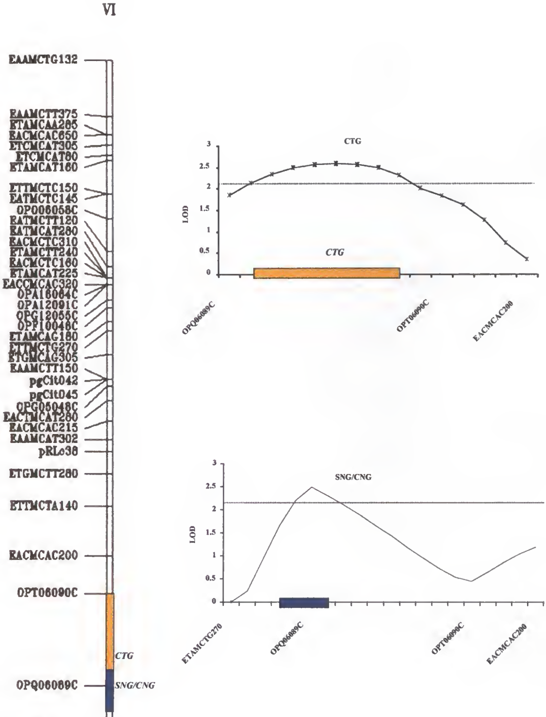

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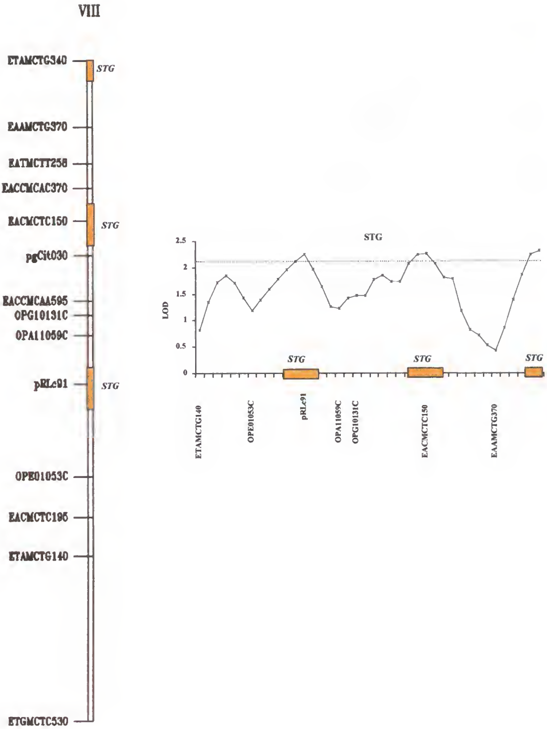

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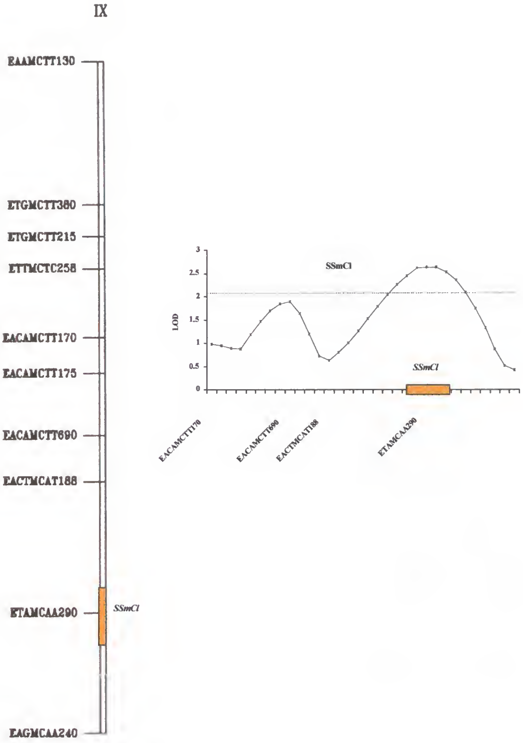

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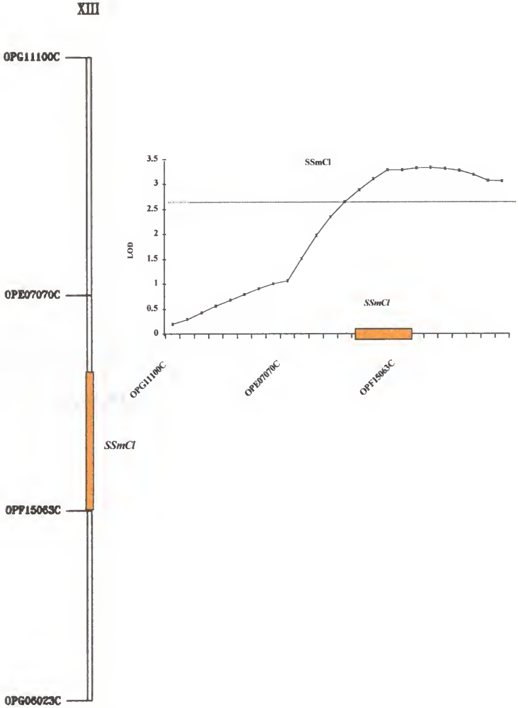

(Figure 4-2 continued)

CHAPTER 5

INTEGRATION OF INDIVIDUAL LINKAGE MAPS FROM THREE INTERGENERIC BACKCROSS POPULATIONS: A CONSENSUS MAP OF A CITRUS DISEASE RESISTANCE GENE REGION

Introduction

Well-saturated genetic maps are required for the molecular manipulation of agronomically important major genes or quantitative trait loci (QTLs). The development of DNA markers, such as RFLP, RAPD, and more recently microsatellites and AFLPs, has resulted in the construction of molecular linkage maps of most crops species, including tree crops like citrus. Several genes have already been cloned using a map-based cloning strategy (Arondel et al., 1992; Giraudat et al., 1992; Chang et al., 1993; Leyser et al., 1993). The gene of interest is positioned initially within a linkage group, relative to mapped molecular markers. Further fine mapping requires the isolation of sufficient recombinants and relies on the density of the existing markers to localize the gene of interest to a small genomic region (Cnops et al., 1996). The closest molecular markers (normally a DNA probe, or markers with single or low copy number) flanking the locus are the starting points for the isolation of overlapping clones from large DNA insert genomic libraries, to construct a contig for chromosome walking towards the gene. With the availability of yeast artificial chromosome (YAC), bacterial artificial chromosome (BAC), and cosmid libraries, large numbers of markers are needed to establish a high-resolution genetic map around the gene of interest. To minimize walking steps and to "land" near the locus of interest, it is critical to isolate a large number of recombinants and to saturate the region around the desired gene with molecular markers (Tanksley et al., 1995). Most recently, another strategy was proposed for map-based gene isolation, called "gene golfing" (Zhang et al., 1997). Gene golfing is primarily based on an integrated high-density genetic and physical genome map, and it is theoretically suited for rapidly cloning a large number of genes known by phenotypes.

Map-based cloning, whether by chromosome walking or gene golfing, relies on a high-density genetic map, especially a map containing many anchor marker across several populations. The AFLP technique generates large numbers of polymorphic molecular markers in a highly reproducible manner, using a minimal amount of plant DNA (Zabeau and Vos 1993; Vos et al., 1995). AFLP is a proven technology to increase marker density around targeted genes and can allow the identification of YACs, BACs, and cosmid clones containing the genes through tightly-linked or co-segregating markers. Recently, the AFLP technique has been applied to construct a high-resolution map around the potato *Gro*1 and *R*1 loci (Ballvora et al., 1995; Meksem et al., 1995). In tomato, after screening 42000 loci for markers tightly linked to the *Cf-9* locus, co-segregating AFLP markers were identified. The two closest AFLP markers were located on a fragment of 15 kb, on each side of the gene (Thomas et al., 1995). AFLP markers co-segregating with the *Arabidopsis TORNADO*1 locus were identified in one chromosomal region, and used to isolate two YAC clones, thus demonstrating successful AFLP-based chromosome landing for the *TRN*1 locus (Cnops et al., 1996).

The availability of a large number of DNA markers has made it possible to obtain fine-scale genetic maps from controlled crosses of many crop species (Tanksley 1992). Most maps are based on a large number of progeny from single experimental crosses (Burr et al., 1988; Tanksley et al., 1992). However, comparative study of certain regions of the chromosomes mapped with common markers in different populations has indicated that, in most cases, the order of molecular markers on the linkage map is identical, although the map distances are different (Börner and Korzun 1998). The interspecific crosses have been used frequently to maximize polymorphism (Eills et al, 1992; Bennetzen and Freeling, 1993; Bradshaw and Stettler, 1995; Kaga et al., 1996). In citrus, intergeneric crosses have been used in mapping studies, and a number of markers appear to be stable across different genetic backgrounds in these intergeneric crosses (Jarrell et al., 1992; Durham et al., 1992; Cai et al., 1994; Gmitter et al., 1996; Deng et al., 1997; Ling et al., in press). Alignment and integration of single-pedigree maps have been reported for several crop species (Beavis and Grant, 1991; Kianian and Quiros, 1992; Hauge et al., 1993; Gentzbittle et al., 1995; Bucci et al., 1997; Börner and Korzun 1998). Procedures for integrating linkage information from different pedigrees are available in computer software packages (Lander and Green, 1987; Stam 1993) and have been widely used across species. The inconsistencies in multi-locus linkage maps based on different pedigrees (Eill et al., 1992; Gmitter et al., 1996; Deng et al., 1997) should be evaluated with regard to the stability of the targeted gene region, and validated by using common markers which are stable across the mapping population as anchors. Thus, the position of a targeted gene can be confirmed by these anchor marker positions.

The *Ctv* gene is one of the most important disease resistance genes in citrus. Map-based cloning of *Ctv* is being pursued by citrus researchers. The *Ctv* gene was mapped first with RAPD markers in four relatively small backcross populations by bulked segregant analysis, and eight RAPD markers linked with *Ctv* were identified (Gmitter et al., 1996). The research work was extended further to convert closely linked RAPD markers into SCAR markers, and a more informative localized linkage map for *Ctv* was generated by mapping additional RAPDs and SCARs (Deng et al., 1997). However, the previous linkage maps were all constructed based on relatively small populations, with no more than seventy individuals in each. For the purposes of map based cloning of *Ctv*, those mapping populations were too small to give an accurate marker position around *Ctv* to facilitate integration with physical maps, or chromosome walking. A larger intergeneric backcross family (9401 population) between *Citrus* and *Poncirus* was produced subsequently, containing nearly 700 offspring. Construction of high-resolution maps based on large populations, for gene cloning, have been reported in many plant species (Martin et al., 1993; Tanksley et al., 1995; Meksem et al., 1995; Thomas et al., 1995; Cnop et al., 1996; Simons et al., 1997). Construction of a high resolution map using a small, selected subset of recombinants from the larger mapping population, based on linkage information from a few marker loci was applied in tomato and *Arabidopsis*;

this approach resulted in a gain in resolution for the selected population, relative to a random population of the same size (Vision and Tanksley 1999). Focusing effort on marker recombinants potentially allows higher throughput at less expense, and is much less time consuming than the conventional mapping approach employing the total large random population. Considering the specific technical difficulties associated with CTV resistance phenotype evaluation in citrus, the large scale phenotype evaluation of a large population was considered to be impractical, for high resolution mapping of the *Ctv* region.

Citrus nematode, *Tylenchulus semipentrans* (Cobb, 1914), is a widespread pest of citrus, that seriously affects the growth of young trees and causes a “slow decline” in mature orchards. Citrus rootstocks that are resistant or highly tolerant to citrus nematode may offer practical and effective means for nematode control (Baines et al., 1960). The inheritance of citrus nematode resistance was studied on the basis of an intergeneric backcross population involved *P. trifoliata* as the resistance source. A major citrus nematode resistance gene region was identified with molecular markers, and it was inherited as a single dominant locus, *Tyr1* (Ling et al., in press). Several molecular markers including RAPDs, SCARs, and an RGC marker linked with *Tyr1* were identified and mapped; all of these markers also were mapped in the R family (see Chapters 3 and 4) and the large family used for high-resolution mapping of the *Ctv* region, as described above (9401population). It should be helpful to integrate the genetic information from different pedigrees (genetic backgrounds) into a consensus linkage map, because information on these two major disease resistance gene regions can be presented in a single linkage map; thus the genetic relationship between these genes from the *Poncirus* genome can be comprehended.

In this chapter, the construction of a high-density consensus linkage map for the region of *Ctv* and *Tyr1* is described. A subset of the large 9401 population was used for the AFLP marker development. The genetic information from three different pedigrees was integrated to produce a localized consensus linkage map. The detailed strategy for targeting *Ctv* by extended bulked segregant analysis will be presented. The methodology of consensus map construction with JoinMap 2.0 will be described in detail, and the analysis of map syntney of the three independent linkage map (groups) will be analyzed. The validation of the consensus map will be discussed.

Materials and Methods

Plant Material

Three intergeneric backcross populations were involved in this mapping study. The data set of the R family, the intergeneric BC1 population derived from cross between 'Thong Dee' pummelo (*Citrus grandis* L.) and USDA 17-40 ['Thong Dee' pummelo (*Citrus grandis* L.) x Pomeroy trifoliate orange (*Poncirus trifoliata*)], which was used to construct a integrated high-density genome map, was used again in this study. A large mapping populationo of 687 hybrids was made by controlled pollination between DPI 4-5

['Nakon' pummelo (*Citrus grandis*)] and USDA 17-47 (a sister seedling of USDA 17-40); this population was designated as the 9401 family. The pollen parent, USDA 17-47, was known to be CTV resistant. The CTV family was grown in the greenhouse under normal management. DNA of each individual progeny was extracted from fully expanded, tender leaves. The standard procedures and PCR conditions were followed for SCAR markers (Deng et al., 1997) and RADP markers (Gmitter et al., 1996), as described previously.

Marker-assisted Selection of the 9401 Population Subset

A total of six *Ctv* molecular markers closely linked to *Ctv*, including five SCARs (Deng et al., 1997) and one RAPD marker (Gmitter et al., 1996), were used to screen the entire 9401 family. The putative *Ctv* gene location was extrapolated according to the relative position of those selected markers (Deng et al., 1997) and information from other mapping experiments. Screening was carried out according to procedures described by Gmitter et al. (1996) and Deng et al. (1997). The individuals possessing all six selected markers, and those without all markers, were excluded from the CTV resistance phenotype study. Presumably, the individuals with and without the six selected markers represented the CTV resistant and CTV susceptible portions of the family, referred to as R and S portions, respectively. The individuals that appeared to be recombinants for those six markers were selected from the large group, and their mature shoots were used later for CTV resistance evaluations. These recombinant individuals constituted a subset of the population in which *Ctv* and its close linked molecular markers (including AFLPs) should segregate. Presumably, the six selected markers still hold close linkage with *Ctv* in the large population.

CTV-resistance Phenotype Evaluations

Seedlings of rough lemon were grown in a temperature-controlled greenhouse (between 15C-28C) and inoculated with Florida CTV strains (either T-66 or T-4) by grafting leaf pieces from infected source trees into the trunk of the rough lemon. The leaves of the rough lemon seedlings were collected 2-3 weeks after inoculation, and tested with a monoclonal antibody (MCA-13), by ELISA according to procedures described by Garnsey et al., (1981, 1987). The rough lemons that gave positive test results were selected and used as rootstocks for the CTV resistance evaluations of the subset hybrids. The buds of each selected subset hybrid (marker recombinant individuals) were grafted onto CTV-infected rough lemon rootstocks. The rootstocks and grafted recombinant hybrid scions were allowed to produce one or two growth flushes before the foliage was harvested for ELISA evaluations. The hybrids designated ELISA-positive (CTV susceptible) were tested positive at least twice, and hybrids designated negative (CTV resistant) were tested negatively at least three times (up to seven times for some plants), with the confirmation of suitable virus challenge based on the positive results on their rough lemon rootstocks. The successful transmittal of CTV virus from rough lemon rootstock to the selected hybrid scion is an important element for determining susceptibility or resistance. Grafted hybrid scions were all subjected to high virus titer challenge by the pathogen (Gmitter et al., 1996).

Screening AFLP Markers by Extended Bulked Segregant Analysis

DNA bulks were constructed based on the results of screening with the six *Ctv*-linked molecular markers, using twenty five individuals from the 9401 population with and twenty five without all of the six markers, respectively. Each pair of DNA bulks was generated by randomly pooling young leaves of five individuals from the putative CTV resistant and susceptible portions of 9401, repectively. DNA was extracted from pooled samples following the extraction procedure described in Chapter 3. A total of 5 pairs of bulks were produced for putative *Ctv*-linked AFLP screening. Bulked DNA samples were processed then by procedures of double restriction enzyme digestion, adapter ligation, and template pre-amplification as described in Chapter 3.

Bulked segregant analysis (Michelmore et al., 1991) was used to screen for polymorphisms between the resistant and susceptible bulks. Although the bulked DNA samples presumably differed in CTV resistance, they should be monomorphic for most other genomic regions. Unique AFLP fragments that were produced in any one of the five resistant bulks were selected as polymorphic fragments and considered to be markers possibly linked with the target gene. Because these DNA bulks are constructed based on the results of marker-assisted selections rather than directed CTV phenotypic evaluations, more than one pair of DNA bulks were used for CTV resistance-linked AFLP marker screening. In this way, the chance of selecting all possible *Ctv*-linked AFLP markers may be increased significantly more than with only one pair of bulks (i.e. to minimize the escape of *Ctv*-linked AFLP markers). Most of the AFLP primers were randomly selected for screening, but the primers that generated AFLP fragments closely linked with *Ctv* in the R family (in Chapter 3) were all used in this DNA bulk screening.

AFLP Marker Screening of the Recombinant Subset Population

DNA was extracted from the young leaves of each individual in the subset population, and AFLP selective templates were generated according to the processes described in Chapter 3. AFLP amplification was carried out with the same PCR conditions described in Chapter 3. The amplification products were analyzed on 6% denaturing polyacrylamide gels. DNA samples were loaded in the gel in the order of parents, five pairs of CTV resistance/susceptible bulks, and individual progenies of the subset population. In each well, 3.5 ul of denatured amplified DNA samples were loaded and run under constant power of 55W for 3 hrs. The gel was transferred to Whatman 2MM paper and vacuum dried for 2 hrs at 90°C on a gel dryer (Bio-Rad). The gel was exposed then to autoradiographic film (BioMax MR, Kodak) at room temperature, overnight to several days.

Data Scoring and Marker Nomenclature

The amplification products were visualized on the autoradiographs. Bands amplified from the pollen parent USDA 17-47 and any one of the five resistance bulks, but absent from the seed parent DPI 4-5 and the correspondent susceptible bulks, and which appeared to be segregating in the population, were scored as dominant markers. The total number of fragments and polymorphisms generated by each primer pair were summarized to reveal the best primer combinations for the amplification. The unreliable, ambiguous bands were not scored. The presence of the AFLP bands implied heterozygotes (Aa) of that locus, and the absence implied homozygotes (aa) of the locus. The gel results were visualized and scored twice independently, as presence/absence of a given band. Heterozygote bands (Aa) and homozygote bands were designated as "h" and "a" in the linkage analysis file respectively. Chi-square tests were performed to verify the segregation ratio of the individual marker. The current mapping data sets consisted of AFLPs, SCARs, RAPDs, and RGCs. AFLP markers were designated according to the name of the restriction enzyme primer combination and the selective nucleotides, followed by the size of amplified fragments. The mapping data of SCARs, RAPDs, and RGCs were obtained from different mapping experiments targetting the *Ctv* gene using the same BC1 population R family (Gmitter et al., 1996; Deng et al., 1997; Deng et al., submitted). The SCAR and RAPD markers were named by beginning with "SC" and "OP" respectively, indicating their primer origin, and followed by the fragment sizes.

RGC markers were named with "RG" and followed by their origin by restriction enzyme digestion.

Linkage and Map Synteny Analysis

The molecular marker data from three different backcross populations were collected. For the 9401 family subset population, the data were generated based on BSA-selected AFLP fragments that segregated in the subset population, and were initially organized into JoinMap 2.0 operational format. Markers were first grouped under relatively high minimum LOD thresholds (LOD = 7.0), then the LOD threshold score was reduced to 5.0 to reach the optimum grouping result, with recombination threshold value of 0.45. The linkage group containing *Ctv* was selected for integration analysis. Marker data from the R family mapping study were adapted and prepared in the same fashion described in Chapter 3, and used to construct a localized linkage map for the CTV resistance gene region, using a threshold LOD score of 2.0. In the same manner, the marker data from the CN resistance study (Ling et al., in press) were transformed and analyzed with JoinMap 2.0. Three independent linkage maps were generated with JoinMap 2.0 and used for map synteny analysis.

The consensus map analysis was based on the joined "pairwise data" of the three independent linkage maps. Each "pairwise data" file was generated with the least restrictive level of the LOD and recombination thresholds to ensure that the maximum number of markers could be used. Markers that failed to meet the requirement of the

LOD and recombination thresholds in the major linkage group were eliminated from the map. The data were analyzed with various ranges of the parameters to reach the optimum grouping and marker orders, including adjustments in various ranges of the threshold LOD scores and threshold of recombination frequencies. The three independently generated linkage maps and the consensus map, all containing the CTV and CN resistance regions, were aligned using common (anchor) markers, *Ctv*, and *Tyr1*, and their flanking marker positions were compared.

Results

Evaluation of CTV Resistance

CTV resistance phenotypes in the 9401 recombinant subset were determined over two years time. At the current time, 63 out of 101 hybrids were successfully evaluated for their reaction to CTV inoculation by ELISA. The individual hybrids with consistent ELISA results were classified as resistant or susceptible. The hybrids that presented positive ELISA test results at least twice were classified as susceptible to CTV, while the resistant ones had a negative ELISA test at least three times. The hybrid plants that did not meet the classification criterion, were all counted as missing data in the linkage analysis at this point. The CTV phenotype results obtained from this selected subset population fit a 1:1 segregation ratio, as expected for a single dominant gene in a backcross population (Table 5-1).

Extended DNA Bulk Screening for Putative *Ctv*-linked AFLPs

Eight pairs of *EcoR*I and *Mse*I primers were used for the bulk screening, and generated thirty polymorphic AFLP fragments between the two bulks, ranging in size from 50bps to 570bps. The RAPD marker OPC19$_{950}$ also was used to screen the whole subset population, and found closely linked with the other six selection markers. There were some monomorphisms observed between some pair of bulks. Each of these 30 AFLP markers was found present in at least one resistance bulk and absent from the correspondent susceptible bulk, and the polymorphisms were confirmed with the parents of this BC1 family (Figure 5-1). Markers that were identified in all of the putative resistance bulks and absent from susceptible bulks were expected to be more promising for linkage with *Ctv*. Ultimately, all of the 30 selected AFLP markers were used to screen individuals in the subset population. AFLP markers EAGMCAC350, EACTMCAA260, and ETTMCTA175, which co-segregated with *Ctv* in the R family were mapped in this selected subset population, but with different linkages to *Ctv* in the subset population. Twenty two BSA-selected AFLP markers in the subset population followed a 1:1 segregation ratio. Segregation distortions were observed for eleven markers (at $p>0.05$) including OPC19950; they were indicated in Table 5-1.

Linkage Analysis and Construction of A Consensus Map

Three localized linkage maps for *Ctv* and *Tyr1* region were generated from three mapping populations with JoinMap 2.0. These independent linkage groups were calculated using relatively higher LOD and recombination frequency thresholds, 0.2 and 0.45 respectively, according to the instruction. The map of linkage group I from the R family (RI map) was calculated with a slightly higher LOD threshold restriction compared to the one constructed in Chapter 4. Two AFLP markers were determined unfit by the calculations and eliminated. A total of eighty-three markers including SCARs, RGCs, RFLPs, AFLPs, and RAPDs were mapped. The genetic coverage for the RI map was 83.6 cM, which was 15.3 cM shorter than the previous one (98.9 cM) in Chapter 4. The RI map served as the major marker contributor for the construction of the consensus map.

For the 9401 family subset population, a total of 39 markers were used for linkage group construction. Under the threshold LOD score of 0.2 and a recombination frequency threshold of 0.45, there were 34 markers successfully mapped into one linkage group with *Ctv*; the map covered 139.8 cM, with average map density of 4.1 cM per marker. Compared with the RI and the CN resistance map, the orders of some common markers were slightly altered in the linkage map of sub9401. Previously identified *Ctv* co-segregating markers were all mapped at various distances from *Ctv*. The CN resistance linked marker, $SCO07_{650}$, was mapped to the distal region of the linkage group, and generated a genetic gap of 21cM. A total of 25 AFLP markers were mapped in the sub9401map; they were placed in between the common markers (Figure 5-2). The alteration of the marker order and the changes in map distance were expected as the number of individuals was increased and a different linkage analysis program was used.

The marker order of the linkage map for the *Tyr1* region was not different from the one generated by MAPMAKER previously (Ling et al., submitted), but the genetic distance between markers was shorter, as expected. SCAR marker $SCT08_{590}$ was newly introduced into linkage analysis and was mapped between $OPAE02_{1600}$ and $OPAJ04_{1400}$. The linkage group covered a genetic distance of 39 cM, which was 27.3 cM shorter than the one generated previously.

The genetic differences calculated by MAPMAKER and JoinMap were also observed in the total genetic map construction (in Chapter 3 and 4). The marker region that harbored *Tyr1* was very well retained, with the same genetic distance between markers as calculated by MAPMAKER.

The linkage data from these three independent mapping populations contained eleven markers that were considered as orthologus markers (anchor markers), common to at least two populations. Three SCAR markers ($SCO07_{650}$, $SCT08_{590}$ and $SCAD08_{1100}$) were common across three populations, and the rest were common across two populations. The 9401 and the R populations have eleven markers in common. The consensus map was constructed based on combined "pairwise data" of each localized linkage map, where all of the genetic information was integrated and represented as marker to marker recombination frequencies under a certain restrictive LOD threshold level. The eleven orthologus markers served as anchors for the integration. The final consensus map was achieved by evaluation array of the mapping results from various levels of the threshold LOD scores, and each mapping result was examined to verify the anchor marker positions compared with the independent linkage groups. The final consensus map resulted from construction under a LOD threshold (0.2) which is 200 fold higher than the recommended level. It consisted of 102 unique markers (45 AFLPs, 3RFLPs, 7 SCARs, 4 RGCs, and 43 RAPDs), and covered 130.7 cM (Table 5-2). The average map density was 1.3 cM for each marker. The final consensus map was presented in Figure 5-4. The orthologus marker order and map distance for the citrus nematode resistance region slightly changed compared to the map previously generated.

The maps of 9401 and the CN resistance linkage map were also integrated, and produced a consensus map CN-9401, for the purpose of synteny analysis. The joined map has a total length of 137.1 cM, and consisted of 45 molecular markers (Figure 5-3). The recombination pairwise data sets of CN and 9401 map were joined under the same LOD and recombination threshold level.

Colinearity Analysis

By comparing the marker positions on each of the three constituent maps with the consensus map, it was found that marker position rearrangements occurred in varying degrees. The colinearity of order among the orthologus markers from the constituent linkage groups were compared; the consensus map represented nearly complete marker colinearity with the CN resistance map, with the exception of $SCT08_{590}$ and $SCO07_{650}$ which mapped to different positions on the consensus map. The shifted position of marker SCT08$_{590}$ and the genetic distance change of SCO07$_{650}$ was indicated in map synteny analysis (Figure 5-2).

Three RFLP markers (pgCito12, pRLc32 and pRLc49) and three RAPD markers (OPQ17064C, OPE05028C and OPA02066C which were mapped between the interval of the three RFLPs) have well retained their colinearity as in the R family map. Relatively higher levels of rearrangement of marker orders were observed for previously mapped markers around *Ctv*. The map distance changes were also widely observed, as expected. For example, in the R family, SCO07$_{650}$ and OPJ07$_{550}$ were mapped 1.1 cM from each other, and SCAM02$_{1000}$, SCAD08$_{1100}$, SCB11$_{700}$ RG11R11, OPC19$_{950}$ and SCT08$_{590}$ co-segregated with *Ctv*. But in the consensus and the sub9401 maps, they were mapped as different loci at various distances away from *Ctv*, and their positions no longer followed the original marker order. SCAD08$_{1100}$ was mapped 3.3 cM away from *Ctv* in sub9401 map, and 1 cM away from *Ctv* in the consensus map. Markers SCAD08$_{1100}$ and SCAM02$_{1000}$ were mapped 16.7 cM away from each other, and were all positioned at one side of *Ctv* on the sub9401 map, but they were mapped 3.3 cM apart in the consensus map and shifted positions to flank *Ctv*. Three AFLP markers (EACTMCAA260, EAGMCAC350 and ETTMCTA175) were co-segregating with *Ctv* in the R family, but on the consensus map they were separated from each other, EACTMCAA260 and EAGMCAC350 flanked *Ctv* within 3 cM. Marker ETTMCTA175 mapped 0.6 cM away from EACTMCAA260 on the consensus map. Marker OPJ07$_{550}$ and SCB11$_{700}$ were 2 cM away in the R family population, but were mapped much farther apart (> 30 cM) in the

9401 population, and mapped 21.4 cM apart on the consensus map. Decreased map distances between some sets of markers were also observed, compared with the correspondent constituent map. For instance, marker RGPt9Hinf mapped 1.5 cM away from *Ctv* on the RI map, but co-segregated with *Ctv* on the consensus map.

In the consensus map, the order of the markers that defined the CN resistance region were retained nearly as they were in the CN resistance linkage map. However, some minor exceptions were found in alteration of some marker positions. $OPO07_{650}$ and $SCO7_{650}$ co-segregated and were closely linked with *Tyr1* in the CN resistance population; however, on the consensus map $OPO07_{650}$ and marker $SCO07_{650}$ were mapped 0.7cM away from each other. RGpt8taqI co-segregated with $SCO07_{650}$ on the consensus map, but it was 3.3 cM away from the $OPO07_{650}$-$SCO07_{650}$ duplex in the CN resistance population.

Most of the AFLP markers on the consensus map were mapped in between the previous known markers, or at the distal ends of the linkage group. Most of the AFLP markers were contributed from the map of the R family. Compared with each constituent map, the change of the marker orders for those common markers was observed mainly in or near the *Ctv* gene region. The markers in the distal regions of the consensus map frequently switched positions when compared with the corresponding constituent maps; however, the corresponding marker clusters still remain.

Discussion

CTV Resistance Phenotype of the Subset Population

Up to the current time, there were about 60% of the individuals in the 9401 population that were successfully phenotyped. The major difficulties for determining the phenotype are the stability of virus transmission from CTV-infected rootstock, and the poor regeneration ability of hybrid scions budded to the infected rootstocks. The CTV infection of the rootstocks was examined by ELISA prior to budding the hybrid scions. Although the rootstocks were inoculated with CTV, in many cases when scions and rootstock were tested, the rootstocks were ELISA negative. These results lead to reconstruction of the rootstock-hybrid scion units for further tests. Another problem encountered was that some hybrid scion buds sprouted poorly, or not at all, on the CTV-infected the rootstock. Whether or not the virus in the rootstock actually caused slow buds sprouting of some hybrids was not investigated, but it seems possible considering the consistent difficulty encountered over the long period of phenotype testing, with high virus titer in the rootstock. Several hybrids yielded ambiguous results with repeated ELISA, and some hybrids had inconsistent results in different times and from different replicates. Inconsistent result among replicates of some individuals may indicate that some virus particles were able to cross the bud union and thus were detected in the scion by ELISA. The same speculation was made by Gmitter et al. (1996) in studying the CTV-resistance phenotype of R family hybrids. Because only 63 selected hybrids had conclusive CTV phenotype test results, the actual map position of *Ctv* relative to the other genetic markers could only be determined approximately. However, with bulked selected *Ctv*-linked markers in the CTV family and complete CTV-resistance phenotype information from R family, a relative position of *Ctv* was determined on this consensus map.

Extended Bulked DNA Screening for CTV Resistance-linked AFLPs

The method used in this study was a novel type of DNA bulk screening. First, marker-assisted selection was conducted to identify individuals that possibly were recombinant for the *Ctv* gene. To ensure the *Ctv* gene recombinant individuals were identified and included in a confident fashion, six previously identified CTV-resistance associated markers were used for the selection, and any of the individual progeny recombinant between any two of the markers were selected.

Five SCARs and one RAPD marker were used for large population (9401 family) screening. All six markers originated from previous studies involving six different families, and almost all of them except marker $SCB11_{700}$ showed universal linkage with *Ctv*. They all were found in USDA 17-47 (the pollen parent of the 9401 population). With such genetic background, the individuals selected by the presence or absence of those selected markers should reflect to a substantial level the CTV resistance or susceptibility. Of course, 100% accuracy for resistance or susceptibility determination should not be expected. In such circumstances, the construction of several distinct pairs of bulks for resistance and susceptibility should provide greater genomic coverage than only one pair of bulks would provide. The rationale behind this approach was that without direct phenotypic information of CTV resistance or susceptibility, great caution should be exercised in determining the position of *Ctv* relative to those selection markers, because there could be some possibility that *Ctv* may be outside of the region flanked by the selection markers. Therefore, four extra pairs of bulks were constructed for Ctv-linked AFLP marker detection.

In the course of bulk screening, several AFLP fragments showed uniform polymorphism between all five pairs of bulks (bands present in all resistance bulks and absent in all susceptible bulks). These fragments were expected to be more closely linked with *Ctv*, and these bands were found to be closely linked with *Ctv* in the linkage analysis. The bands that were present in the resistant parent and any one of the five resistance bulks were also recorded and used to screen individuals of the subset 9401 population. The putative *Ctv*-linked AFLPs were segregated with *Ctv* in the subset 9401 population. This indicate that the subset 9401 still can be considered as a random population for many of the those AFLP loci. Monomorphism found between some pair of bulks implied that some individuals in the DNA bulk may be recombinant for *Ctv* and the selection markers.

Linkage Analysis and the Consensus Map

The linkage analysis was conducted for each population with JoinMap 2.0. For the R family, linkage analysis was conducted only for linkage group I, because that is the linkage group harboring *Ctv* and CN resistance-linked markers. All of the calculations for different maps were under relatively higher LOD strength, which eliminated troublesome markers from the consensus map construction. The markers that mapped closely linked to or co-segregating with *Ctv* in the RI map were analyzed in the 9401 population; none of them co-segregated with *Ctv* and the genetic distances between markers were farther apart. The markers EACTMCAA260 and EAGMCAC350 co-segregated with *Ctv* in the RI map, but they mapped farther apart in sub9401 than expected. Marker EAGMCAC350 was closer to *Ctv* than EACTMCAA260 in the linkage map of sub9401, but these two markers mapped within 5cM of each other and flanked *Ctv* in the consensus map. The inconsistency of the marker position in different constituent maps reflects unequal recombination in the different populations, which may be associated with the different size of the populations and/or the internal genomic arrangements of the intergeneric hybrid parents. Uneven distribution of the markers in the intergeneric hybrid genome background may have contributed also to inconsistent colinearity. The uneven distribution of RFLP markers between *Poncirus* and *Citrus* was reported by Jarrell et al. (1992).

$SCAD08_{1100}$ in both the sub9401 and consensus maps, was placed close to *Ctv*, 3.3 cM and 1cM away respectively. In the R family map, $SCAD08_{1100}$ co-segregated with

Ctv. It is expected that genetic distance would increase with increasing of the population size. The stability of $SCAD08_{1100}$ relative to *Ctv* indicated a genomic region universally associated with the gene across different populations, which makes $SCAD08_{1100}$ likely to be the candidate marker valuable for marker-assisted selection in populations which have *Poncirus* involved in their pedigree.

$SCO07_{650}$ was mapped to the distal region of the linkage group in sub9401 population, with a large gap (21 cM). This result may reflect non-random sampling of the genome resulting from an under-representation of markers in this region, but it also due to the limited number of markers available in this subset population. The gap may also be the result of cytogenetic phenomenon, i.e. the proximity of centromeres, or the uneven distribution of the recombination events along the chromosome (Tanksley et al., 1992). The gap between $SCO07_{650}$ and the rest of the linkage group in the consensus map supports the suggestion made by Deng et al (1999, submitted), that chromosomal rearrangements in the *Ctv-Tyr1* region in different intergeneric crosses may be the cause of abnormal behavior of their linked markers. The generation of gaps were reported also in consensus map construction of pine (Sewell et al., 1999), rye (Börner and Korzun, 1998) and maize (Beavis and Grant, 1991). The genetic distance differences among the three maps for $SCO07_{650}$ and RGpt8taqI may reflect the complexity of this genomic region as a resistance gene cluster region resulting from intergeneric crosses.

Most of the informative markers were contributed to the consensus map by the R family. It has been pointed out, that given a large number of populations, any given region of the genome may be associated with unequal recombination in at least one population (Beavis and Grant, 1991). A possible reason for unequal recombination with a common marker set in different populations was suggested long ago; linkage maps with a common set of genetic markers in different populations may not be equivalent, because individual markers fail to exhibit Mendelian segregation or because recombination is not homogenous among the population (Morton 1955). In the study reported herein, most of the markers followed Mendelian segregation so, it is more likely that the recombination frequencies varied within and among the populations. These differences resulted in unequal map distances within the different populations and the consensus map. Furthermore, JoinMap 2.0 calculates the composite map distances based on the predetermined marker recombination pairwise data from each constituent population, so marker order and map distance have to be adjusted by the program itself to reach the most appropriate configuration under the given threshold levels of LOD and recombination frequency. Together, these various factors result in inconsistent marker orders and map distances between the consensus map and the constituent maps. The anchor markers that are common across the different populations play an important role in the integration of different maps. With anchor markers, the relative position of all other marker loci can be extrapolated. Thus, as more anchor markers are available, the more accurate the consensus map will be. There were ten markers (9 AFLPs, and 1 RAPD) displaying distorted segregation in the 9401 population, and all 9 AFLP markers were distorted toward the recurrent parent. This result was very similar to that found in the R family map. The same distortion toward the recurrent parents was reported in previous citrus mapping studies (Durham et al., 1992; Jarrell et al., 1992; Gmitter et al., 1996). These distorted segregation ratios are somehow related with the population structure, i.e. the intergeneric cross population. Presumably all the AFLP markers were associated with *Ctv* to various degrees, thus the distortion also indicates some alterations of this genomic region.

The total linkage group length of the consensus map was 130.7 cM, compared with 139.8 cM for sub9401 map, 39 cM for the CN resistance linkage map, and 83.6 cM for the R family linkage group I. From this comparison, it is possible to conclude that the consensus map has not been saturated by markers. When constructing the total genome map of the R family (with 63 individuals), linkage group I (with 83 markers) was not fully saturated; and for the 9401 and CN resistance population, these studies focused only on the two disease resistance regions, respectively. Thus, the integration was focused mainly on these regions rather than the whole chromosome. Furthermore, the fact that map lengths of the consensus and 9401 maps were not much different also supported this conclusion. The same conclusion was reached in developing a consensus map of sunflower (Gentzbittel et al., 1995) where the constituent maps were not saturated, the consensus map could be of greater length.

Conclusion

Not only can the differences among the three maps be attributed to the use of different polymorphic markers, or at least different numbers of polymorphic markers, but also to the difference in the recombination rates in different populations for those orthologous markers. The unequal recombination among different populations raises the question of whether or not the data should be pooled and a consensus map constructed. Given a large number of populations, any genomic region may be associated with unequal recombination in at least one population, compared with the others. Thus, the actual map for an individual population will deviate from the consensus map significantly. Despite this major weakness, the consensus map can be useful, as a reference in planning experiments that require genetic markers to be dispersed uniformly throughout the genome and to compare quantitative trait loci identified in different genetic backgrounds.

The primary goal for the construction of this consensus linkage map was to place, relative to one another, as many genetic markers as possible onto a single linkage map. By integrating all the genetic information from different populations, and because the traits of interest were not completely evaluated phenotypically in every population, very accurate mapping results cannot be expected. Therefore, the concern was more towards obtaining a general order and distance among the markers, rather than fine resolution of the order and the distance.

Through the research reported here, the integration of genetic information obtained from different genetic backgrounds was successfully conducted. Pre-conducted marker-assisted selection was used to select individuals of a large intergeneric backcross population, and a subset mapping population was established for CTV-resistance phenotype evaluation. Marker-based extended bulk segregant analysis was used to identify AFLP markers linked with CTV resistance within the subset population of the marker recombinant individuals, and a local linkage map of the *Ctv* region was constructed. The general idea was to use selected marker-recombinant individuals for mapping CTV resistance, because phenotype evaluation is very difficult to conduct with a large segregating population. Citrus nematode resistance is another complex trait, and it is often difficult to distinguish among the individuals; valid resistance phenotype evaluation consumes a lot of time and labor. Taking the advantage of previously identified resistance gene regions, genetic information of CN and CTV resistance were integrated into a single consensus linkage group, along with the well-mapped R family, which had nearly complete CTV resistance phenotype evaluation.

The reliability of the AFLP markers was confirmed again on the basis of their inheritance in the 9401 population. The methodology of extended bulk segregant analysis based on marker-assisted selection was first explored for CTV resistance-linked AFLP fragments. The orthologus markers among these three backcross populations were examined for the colinearity of the different genomic regions, and the possible genomic region alterations related to the different crosses were extrapolated. In most cases, the order of molecular markers on the linkage maps from these three populations were nearly identical with few exceptions. Although there may be some uncertainty in the order of mapping points extrapolated from the different mapping populations, the consensus linkage map presented will enable potential users to choose markers in the region of interest for gene tagging. The consensus map methodology will be useful for the collection of genetic information of other traits in crosses of other genetic backgrounds.

Table 5-1: The Chi-square tests for the goodness of fit of segregation ratios of markers identified in the 9401 subset population.

Marker	Present	Absent	Expect ratio	Chi-square
Ctv	32	31	1:1	0.008
EACCMCAG550	40	58	1:1	1.65
EACCMCAG520	59	39	1:1	2.04
EACCMCAG390	30	68	1:1	7.36 **
EACCMCTG570	46	54	1:1	0.32
EACCMCTG320	40	60	1:1	2.0
EACTMCAA400	41	55	1:1	1.02
EACTMCAA260	51	47	1:1	0.08
EACTMCAA340	19	28	1:1	0.86
EACTMCAA225	15	32	1:1	3.07 *
EAGMCAC350	46	55	1:1	0.40
EAGMCAC320	37	64	1:1	3.60 **
EAGMCAC240	60	41	1:1	1.79
EAGMCAC230	48	53	1:1	0.12
EAGMCAC170	49	52	1:1	0.04
EAGMCAC130	50	51	1:1	0.005
EAGMCTT560	46	55	1:1	0.40
EAGMCTT380	46	55	1:1	0.40
EAGMCTT320	40	61	1:1	2.18
EAGMCTT220	48	53	1:1	0.12
EAGMCTT200	42	59	1:1	1.43
EAGMCTT190	38	63	1:1	3.09 *
EAGMCTT170	37	64	1:1	3.60 **
EACMCAG430	36	65	1:1	4.16 **
EACMCAG380	42	59	1:1	1.43
EACMCAG260	15	35	1:1	4.0 **
EACMCAG250	37	64	1:1	3.60 **
EACMCAG240	30	21	1:1	0.79
EACMCAG225	24	27	1:1	0.09
EACMCAG170	43	58	1:1	1.11
EACMCAG180	39	62	1:1	2.61
SCO07650	52	49	1:1	0.04
SCAD081100	48	53	1:1	0.12
SCAM02980	51	50	1:1	0.005
SCT08590	55	46	1:1	0.40
SCB11700	47	52	1:1	0.13
OPJ07550	47	54	1:1	0.24
OPW18600	55	46	1:1	0.4
OPC19950	74	26	1:1	11.52 **

* and ** indicate significant deviation from expected 1:1 ratio at $P = 0.1$ and 0.05, respectively.

Table 5-2: Comparison of the linkage map coverage by each of the localized linkage maps from different mapping populations and consensus maps. All the maps were constructed by JoinMap.

Localized linkage maps	Total markers	Mapped markers [a]	Map coverage (cM) [b]
R family linkage group I	85	83 (97)	83.6
CN resistance	15	15 (100)	39.0
9401 subset CTV region	39	34 (87)	139.8
CN resistance/9401 consensus	46	45 (98)	137.1
Final consensus map	105	103 (98)	130.7

[a] Percentage of mapped markers in parentheses.

[b] Map units in centiMorgans using Kosambi mapping function.

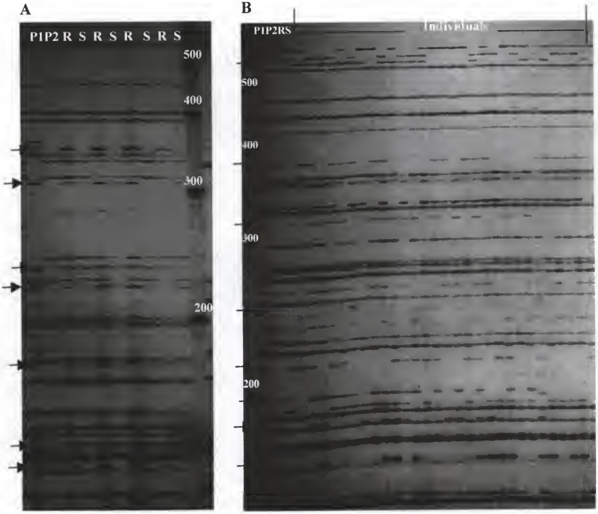

Figure 5-1(AB). A: DNA bulk screening for putative AFLP markers linked with CTV resistance. The AFLP fragments were amplified by primer combination EAGMCTT. The CTV resistant parent P1 = USDA 17-47 ['Thong Dee' pummelo (*C. grandis*) x pomeroy (*P. trifoliata*)], the CTV susceptible parent P2 = DPI 4-5 ['Nakon' pummelo (*C. grandis*)], and four pairs of CTV resistance and susceptible bulks were listed. The polymorphic AFLP markers were indicated by arrowheads. B: Autoradiogram obtained with primer combination EAGMCTT showing segregation of alleles revealed by AFLP markers. The parental lanes and DNA bulks are based on marker-assisted selection results. AFLP fragment size was indicated by ^{33}P-labeled DNA size markers.

Figure 5-2: The localized linkage maps from three different intergeneric backcross populations. The chromosome title RI, 9401sub and CN represent the R family linkage group I, the sub9401 map, and the CN resistance linkage map, respectively. The common markers across at least two populations were indicated with *asterisks*.

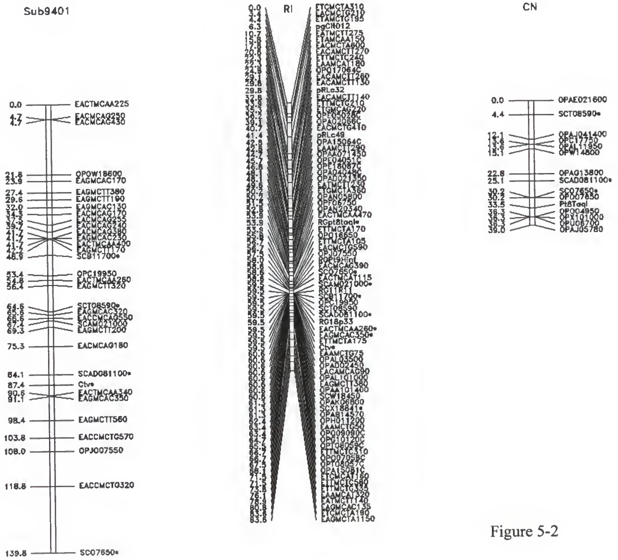
Sub9401
0.0 EACTMCAA225
4.7 EACMCAG250
4.7 EACMCAG430
21.8 OPOW18600
23.9 EAGMCAC170
27.4 EAGMCTT380
29.6 EAGMCTT190
32.0 EAGMCAC130
34.3 EACMCAG170
37.5 EACMCAG255
39.7 EACMCAG240
41.7 EACMCAG380
41.7 EAGMCAC230
41.7 EACTMCAA400
42.2 EAGMCTT170
46.9 SCB11700•
53.4 OPC19950
54.8 EACTMCAA260
56.4 EAGMCTT320
64.6 SCT08590•
65.6 EAGMCAC320
66.6 EACCMCAG550
67.4 SCAM021000
69.3 EAGMCTT200
75.3 EACMCAG180
84.1 SCAD081100•
87.4 Ctv•
90.6 EACTMCAA340
91.1 EAGMCAC350
98.4 EAGMCTT560
103.8 EACCMCTG570
108.0 OPJ007550
118.8 EACCMCTG320
139.8 SC07650•
RI
ETCMCTA310
EACMCTG210
ETAMCTG195
pgCIt012
EATMCTT275
ETAMCAA150
EACMCTA600
EACAMCTT270
ETTMCTC240
EAAMCAT180
OPQ17064C
EACAMCTT260
EACAMCTT130
pRLc32
EACAMCTT140
ETTMCTG210
ETGMCAG220
OPE05028C
OPA02066C
EACMCTG410
pRLc49
OPA15064C
EAAMCTT290
OPAA071450
OPE04051C
OPE18087C
OPA04048C
OPAD021350
EATMCTT230
ETGMCTA360
OPAN05800
OPF06750
OPAN20340
EACTMCAA470
RGpt8taql•
ETTMCTA170
OPO16650
ETTMCTA105
EACMCTG590
OPJ07550
RGPt9Hinf
EACMCAG390
SC07650•
EACTMCAT115
SCAM021000•
RG11R11
SCB11700•
OPC19950
SCT08590
SCAD081100•
RG18p33
EACTMCAA260•
EAGMCAC350•
ETTMCTA175
Ctv•
EAAMCTG75
OPAL03500
OPAD02450
EACAMCAG90
OPAL101000
EAGMCTT380
OPAA101400
SCW18450
OPAK06800
SCX18841•
OPAB14570
OPH011200
EAAMCTG50
OPQ09090C
OPG10120C
OPT08059C
ETTMCTC310
OPO07058C
OPT08057C
OPA15081C
ETCMCAT160
ETTMCTC580
ETTMCTG335
EAAMCAT320
EATMCTT140
EAGMCAC135
ETCMCTA190
EAGMCTA1150
0.0
83.8
CN
0.0 OPAE021600
4.4 SCT08590•
12.1 OPAJ041400
13.6 OPC17750
13.6 OPAL11950
15.1 OPW14800
22.8 OPAG13800
25.1 SCAD081100•
30.2 SC07650•
30.2 OPD07650
33.5 Pt8TaqI
36.3 OPO04950
36.3 OPX101000
36.3 OPU06700
39.0 OPAJ05780

Figure 5-2

Figure 5-3: The consensus map by integration of the CN resistance linkage map and the sub9401 linkage map. The consensus map was indicated with the chromosome title *9401sub/CN*. The common markers were indicated with *asterisks*.

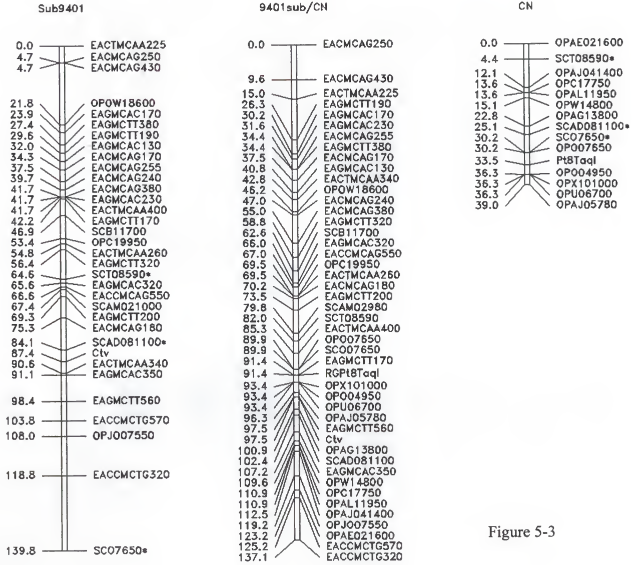
Sub9401
0.0 EACTMCAA225
4.7 EACMCAG250
4.7 EACMCAG430
21.8 OPOW18600
23.9 EAGMCAC170
27.4 EAGMCTT380
29.6 EAGMCTT190
32.0 EAGMCAC130
34.3 EACMCAG170
37.5 EACMCAG255
39.7 EACMCAG240
41.7 EACMCAG380
41.7 EAGMCAC230
41.7 EACTMCAA400
42.2 EAGMCTT170
46.9 SCB11700
53.4 OPC19950
54.8 EACTMCAA260
56.4 EAGMCTT320
64.6 SCT08590*
65.6 EAGMCAC320
66.6 EACCMCAG550
67.4 SCAM021000
69.3 EAGMCTT200
75.3 EACMCAG180
84.1 SCAD081100*
87.4 Ctv
90.6 EACTMCAA340
91.1 EAGMCAC350
98.4 EAGMCTT560
103.8 EACCMCTG570
108.0 OPJ007550
118.8 EACCMCTG320
139.8 SC07650*
9401sub/CN
0.0 EACMCAG250
9.6 EACMCAG430
15.0 EACTMCAA225
26.3 EAGMCTT190
30.2 EAGMCAC170
31.6 EAGMCAC230
34.4 EACMCAG255
34.4 EAGMCTT380
37.5 EACMCAG170
40.8 EAGMCAC130
42.8 EACTMCAA340
46.2 OPOW18600
47.0 EACMCAG240
55.0 EACMCAG380
58.8 EAGMCTT320
62.6 SCB11700
66.0 EAGMCAC320
67.0 EACCMCAG550
69.5 OPC19950
69.5 EACTMCAA260
70.2 EACMCAG180
73.5 EAGMCTT200
79.8 SCAM02980
82.0 SCT08590
85.3 EACTMCAA400
89.9 OP007650
89.9 SC007650
91.4 EAGMCTT170
91.4 RGPt8TaqI
93.4 OPX101000
93.4 OP004950
93.4 OPU06700
96.3 OPAJ05780
97.5 EAGMCTT560
97.5 Ctv
100.9 OPAG13800
102.4 SCAD081100
107.2 EAGMCAC350
109.6 OPW14800
110.9 OPC17750
110.9 OPAL11950
112.5 OPAJ041400
119.2 OPJ007550
123.2 OPAE021600
125.2 EACCMCTG570
137.1 EACCMCTG320
CN
0.0 OPAE021600
4.4 SCT08590*
12.1 OPAJ041400
13.6 OPC17750
13.6 OPAL11950
15.1 OPW14800
22.8 OPAG13800
25.1 SCAD081100*
30.2 SC07650*
30.2 OP007650
33.5 Pt8TaqI
36.3 OP004950
36.3 OPX101000
36.3 OPU06700
39.0 OPAJ05780

Figure 5-3

Figure 5-4: The final consensus map generated by integration of maps from three different intergeneric backcross populations. The map was separated into two parts. The citrus nematode resistance gene region was indicated by its closely linked marker SCO7650 and RGPt8TaqI.

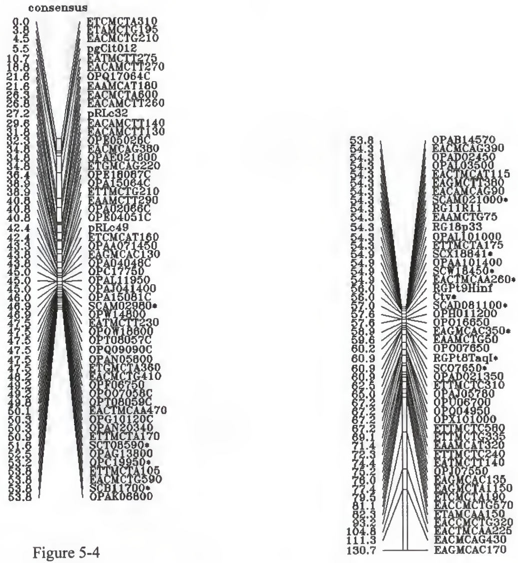
consensus
0.0 ETCMCTA310
3.8 ETAMCTG195
4.5 EACMCTG210
5.5 pgCit012
10.7 EATMCTT275
18.8 EACAMCTT270
21.6 OPQ17064C
21.6 EAAMCAT180
26.3 EACMCTA600
26.8 EACAMCTT260
27.2 pRLc32
29.6 EACAMCTT140
31.8 EACAMCTT130
32.3 OPE05026C
34.8 EACMCAG380
34.8 OPAE021600
34.8 ETGMCAG220
36.4 OPE18087C
38.9 OPA15064C
38.9 ETTMCTG210
40.8 EAAMCTT290
40.8 OPA02066C
40.8 OPE04051C
42.4 pRLc49
42.4 ETCMCAT160
43.3 OPAA071450
43.8 EAGMCAC130
43.8 OPA04048C
45.0 OPC17750
45.0 OPAL11950
45.0 OPAJ041400
46.0 OPA15081C
46.9 SCAM02980•
46.9 OPW14800
47.5 EATMCTT230
47.5 OPOW18600
47.5 OPT08057C
47.5 OPQ09090C
47.5 OPAN05800
47.5 ETGMCTA360
48.2 EACMCTG410
49.2 OPF06750
49.2 OPO07058C
49.8 OPT08059C
50.1 EACTMCAA470
50.3 OPG10120C
50.3 OPAN20340
50.9 ETTMCTA170
51.6 SCT08590•
52.5 OPAG13800
53.2 OPC19950•
53.8 ETTMCTA105
53.8 EACMCTG590
53.8 SCB11700•
53.8 OPAK06800
53.8 OPAB14570
54.3 EACMCAG390
54.3 OPAD02450
54.3 OPAL03500
54.3 EACTMCAT115
54.3 EAGMCTT380
54.3 EACAMCAG90
54.3 SCAM021000•
54.3 RG11R11
54.3 EAAMCTG75
54.3 RG18p33
54.3 OPAL101000
54.3 ETTMCTA175
54.9 SCX18841•
54.9 OPAA101400
54.9 SCW18450•
54.9 EACTMCAA260•
56.0 RGPt9Hinf
56.0 Ctv•
57.0 SCAD081100•
57.6 OPH011200
57.6 OPO16650
58.9 EAGMCAC350•
59.6 EAAMCTG50
60.2 OPO07650
60.9 RGPt8TaqI•
60.9 SCO7650•
60.9 OPAD021350
62.5 ETTMCTC310
65.0 OPAJ05780
67.2 OPU06700
67.2 OPO04950
67.2 OPX101000
67.2 ETTMCTC580
69.1 ETTMCTG335
71.4 EAAMCAT320
72.3 ETTMCTC240
74.4 EATMCTT140
75.2 OPJ07550
76.0 EAGMCAC135
77.4 EAGMCTA1150
79.5 ETCMCTA190
81.1 EACCMCTG570
82.3 ETAMCAA150
93.2 EACCMCTG320
104.8 EACTMCAA225
111.3 EACMCAG430
130.7 EAGMCAC170

Figure 5-4

CHAPTER 6
SUMMARY

Construction of A Total Citrus Genome Map with AFLP Markers

AFLP is a rapidly emerging, molecular marker technique which is gaining widespread application in many areas of genetic studies, including genetic linkage mapping and gene cloning through a genome-based approach. By applying AFLP techniques to the construction of the citrus genome map, it was possible to characterize the AFLP profiles for *Citrus* and the related genus *Poncirus*. Useful information of AFLP loci in these genomes was obtained for further studies. The amplification ability of AFLP loci in the citrus genome, and the levels of polymorphism of the intergeneric backcross population were demonstrated. The potential uses as reliable molecular markers for high resolution mapping of citrus, and the application to citrus genetics and breeding were discussed. The map density of the citrus genome was increased to a new level, and it can serve as the framework for further genetic and QTL analysis.

The development of AFLP markers in citrus was fully explored, and the potential of using AFLP markers for citrus genetic mapping has been demonstrated. The AFLP stability, reproducibility, and efficiency were confirmed further in this study. By using AFLPs, it was possible to generate a higher density map than possible with the other marker systems that had been used previously for citrus mapping. Furthermore, a substantial number of markers were developed for linkage mapping analysis, even when only small mapping populations were available.

Based on the information provided on linkage group I, the order of pre-determined RAPD and SCAR loci remained unchanged, although the entire linkage group was densely populated with many new AFLP markers. However, the number of linkage groups generated in this study was greater than the haploid chromosome number of citrus ($n = 9$), and many minor linkage groups were also identified. It is likely that these minor linkage groups will join with the larger linkage groups with identification of additional markers. In addition, the total map length was less than the estimated citrus genome length. Therefore, it was obvious that the citrus genome has not yet been saturated with molecular markers.

Like RAPD markers, some AFLPs exhibited poor resolution and instability was also observed. Some polymorphic AFLPs were excluded from linkage analysis because of their instability. Therefore, making the best effort to improve technically the gel resolution is very critical and necessary, and individual AFLP markers must be evaluated carefully prior to use for other mapping purposes.

There were 89 AFLP markers detected in this study that were heterozygous in the recurrent parent *C. grandis*. This finding was nearly 50% higher than with the RAPD marker system. By combining data from these two experiments, it should be possible to generate a genetic map for *C. grandis* as well, though this was not done. In this experiment, only 40% of the possible primer combinations available were used, so there remains great potential for the discovery of new markers. It will be advisable in the future to screen more primer combinations, including the utilization of different restriction enzyme systems. AFLP markers used in this study were all manually scored as dominant type markers, but it is possible that some co-dominant type markers could be scored with suitable software programs, which would make this technique more informative.

The AFLP marker system can be very helpful in searching for new gene regions and QTLs, by increasing the marker density on maps in an accelerated fashion. A high-density citrus linkage map can be used to identify genes that are horticulturally important, CTV resistance, *Phytophthora* tolerance, resistance to citrus nematodes, and QTLs for cold hardiness, salt tolerance, tree vigor, fruit size, quality, etc. AFLP markers closely linked to the important genes and major QTLs can be converted to SCAR markers to facilitate marker-assisted selections in simple and relatively inexpensive PCR assays.

Integration of the AFLP Citrus Genome Map with Previous Citrus Maps and QTL Analysis of Selected Salt Tolerance-related Traits

In this study, two sets of data that included several types of molecular markers were integrated successfully into a total citrus genome map. Although the total genetic coverage of this composite citrus map was shorter than the one previously generated (Cai et al., 1994), all of the map regions were more densely populated with AFLP markers. Creating a high-density genomic region by integrating existing genetic information with AFLP markers was proved feasible. The *Ctv* region, made up of several closely linked markers, remained stable when compared with AFLPs and other types of markers identified in previous experiments.

The stability of some important markers was evaluated when different types of markers were introduced into the same genomic region. The merger of previous linkage groups into a single larger linkage group, and the breaking and rearrangement of some previous linkage groups, indicates the need for more markers for linkage analysis and increasing population size for a saturated citrus genome map. Map synteny analysis reflected differences of the calculations for linkage determination by MapMaker and JoinMap mapping programs. JoinMap produced shorter linkage groups than MapMaker.

The integration of two genetic maps together created a high-density map base for QTL analysis. The advantage of a high-density map for QTL analysis in citrus was demonstrated by more accurate QTL locations relative to markers. The QTL analysis of salt tolerance- and growth-related traits revealed certain levels of citrus genome complexity for the salt tolerance-related regions. The possible linkage relationships between some of the QTLs were suggested, and the possible interactions between QTLs were reflected by the QTL peak values, the percentage of phenotypic variance explained by individual QTLs, and the additive effects in both positive and negative sense. This study was the first to relate AFLP markers with QTL traits, in conjunction with other types of markers in citrus.

The JoinMap 2.0 compatible QTL analysis program is suitable for analysis of multiple population types and can be used to conduct QTL analysis in an integrated fashion. This is important for citrus, because many open pollinated or inter-related populations are available in the field, and many of their traits can be measured for the QTL analysis. This program is very useful for identifying conserved loci, markers, and gene locations for traits of horticultural interest, across different populations in citrus. It would be more beneficial if cDNA clone markers and characterized BAC clones for qualitative alleles could be identified in linkage with QTL regions.

Construction of Consensus Localized Linkage Maps for Disease Resistance Gene Regions with Different Population Sources in Citrus

Consensus maps can be used as references in planning experiments that require genetic markers to be dispersed uniformly throughout the genome and to compare quantitative trait loci identified in different genetic backgrounds. The primary goal for the construction of this consensus linkage map was to place, relative to one another, as many genetic markers as possible onto a single linkage map. By integrating all of the genetic information from different populations, including traits that were not phenotypically evaluated in every population, however, very accurate mapping results should not be expected. Therefore, the concern was more towards obtaining a general order and distance among the markers, rather than the fine resolution of order and distance.

Through the research reported here, an integration of genetic information obtained from different genetic backgrounds was conducted successfully. Pre-conducted marker-assisted selection was used to select individuals from a large intergeneric backcross population, and an subset mapping population was established for CTV resistance phenotype evaluation based on the results of marker-assisted selection. A marker-based extended bulk segregant analysis was used to identify AFLP markers linked with CTV resistance within the subset of the population constituted of the marker recombinant individuals; through this approach a local linkage map for the CTV resistance gene (*Ctv*) was constructed. The general idea of using a marker selected subset population for mapping was developed because it is very difficult to conduct CTV resistance phenotype evaluation for all individuals of a large segregating population. Citrus nematode resistance trait is a complex trait, and it is often difficult to distinguish among the individuals. Previously obtained genetic information of CN resistance and CTV resistance were integrated into a single consensus linkage group, along with information from a third family (the R family, which had nearly complete CTV resistance phenotype evaluation).

The orthologus markers among these three backcross populations were examined for their colinearity in different genomic regions, and the possible genomic region alterations among the different crosses were examined. In most cases, the order of anchor markers on the linkage maps from these three populations meet was conserved, with few exceptions. Although there was some uncertainty in the order of mapping points extrapolated from the different mapping populations, the consensus linkage map presented here will enable potential users to choose markers in the region of interest for gene tagging. The consensus map methodology will be useful for the collection of genetic information for other traits, in a wide range of genetic backgrounds in citrus.

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